UNIVERSITY OF LONDON THESIS

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OCULAR MONITORING IN IMMUNOSUPPRESSED PATIENTS AND QUANTIFICATION OF IMMUNOSUPPRESSION BY ASSESSMENT OF INTRACELLULAR CYTOKINES

A thesis presented for the degree of Doctor of Medicine, Faculty of Medicine, University of London

By

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ABSTRACT

Although laboratory indices, such as drug levels, are in routine use for monitoring immunosuppression, these do not always correlate well with an individual's risk of toxicity. In practice individual organs are monitored for toxicity with a combination of laboratory and clinical methods. These methods are limited by the fact that one has to await compromise before making therapeutic changes and there are often idiosyncrasies in the way individuals respond.

Ocular complications can be sight threatening and many have proposed routine ocular monitoring in immunosuppressed patients. This study prospectively monitored a cohort of patients receiving high levels of immunosuppression for the prevention of rejection of heart, lung and heart-lung transplants for the development of ocular complications and to assess ocular morbidity.

Specific surrogate markers that gave more information about the level of immunosuppression in a particular patient would improve patient care. Cytokines have the advantage of being directly generated by the immune response and offer promise as surrogate markers. Various, and not always consistent, cytokine profiles have been described for a number of conditions. Flow cytometry with intracellular cytokine staining allows quantification of the amount of cytokine present. These studies used this technique to describe the cytokine profile and changes in cytokine profile seen in patients during treatment for autoimmune uveitis and in patients with human immunodeficiency virus (HIV) receiving combination anti-retroviral therapy (ART).

These studies emphasized the importance of prompt and careful clinical examination in the presence of clinical symptoms but did not support routine screening of patients on high levels of immunosuppression. Accurate measurements of interleukin-2 (IL-2) and interferon-gamma (IFNγ) in patients with uveitis did not correlate well with disease activity. In contrast patients with different HIV profiles did show measurably different Th1 cytokine expression providing information on the T cell deficits that persist despite treatment with ART.

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ABBREVIATIONS

5-FU	5-Fluorouracil
6-MP	6-Mercaptopurine
6-TG	6-Thioguanine
ACTH	Adrenocorticotropic Hormone
AIDS	Acquired Immunodeficiency Syndrome
AP-1	Activating Protein 1
APC	Antigen Presenting Cells
ARN	Acute Retinal Necrosis
ART	Anti-retroviral Therapy
ATP	Adenosine Triphosphate
Aza	Azathioprine
BD	Behcet's Disease
BFA	Brefeldin A
CAM	Cellular Adhesion Molecules
CD	Clusters of Differentiation
cDNA	Copy DNA
CMV	Cytomegalovirus
CRVO	Central Retinal Vein Occlusion
CsA	Cyclosporine A
CSF	Colony Stimulating Factors
CSF	Cerebrospinal Fluid
CSR	Central Serous Chorioretinopathy
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
EBV	Epstein Barr Virus

ELISA	Enzyme Linked Immunosorbent Assay
FACS	Flourescence Activated Cell Sorter
FBC	Full Blood Count
FHC	Fuch's Heterochromic Cyclitis
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GTP	Guanosine Triphosphate
HAART	Highly Active Anti-retroviral Therapy
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HPRT	Hypoxanthine Phosphoribosyl Transferase
HSV	Herpes Simplex Virus
ICAM	Intercellular Adhesion Molecules
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin 2 Receptor
KS	Kaposi's Sarcoma
LFA	Lymphocyte Function Antigen
LFT	Liver Function Test
MAB	Monoclonal Antibodies
MHC	Major Histocompatibility Complex
MIF	Migration Inhibitory Factor
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
MRI	Magnetic Resonance Imaging
N/A	Not applied
NF-AT	Nuclear Factor of Activated T Cells

NF-kB	Nuclear Factor Kappa B
NK	Natural Killer Cells
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PKC	Protein Kinase C
PMA	Phorbol-12-Myristate-13-Acetate
PML	Progressive Multifocal Leukoencephalopathy
PMT	Photomultiplier Tubes
PSCLO	Posterior Subcapsular Lens Opacity
PTLD	Post Transplantation Lymphoproliferative Disorder
RNA	Ribonucleic Acid
RPE	Retinal Pigment Epithelium
STAT	Signal Transducer and Activator of Transcription
Tac	Tacrolimus
TcR	T Cell Receptor
Th0	T Helper 0
Th1	T Helper 1
Th2	T Helper 2
TNF	Tumour Necrosis Factor
TPMT	Thiopurine S-Methyltransferase
U&E	Urea and Electrolytes
VKH	Vogt-Koyanagi-Harada Disease
VZV	Varicella Zoster Virus

HYPOTHESES AND OBJECTIVES

HYPOTHESES

1. Patients on high levels of immunosuppression have unrecognized, asymptomatic eye disease which may be sight threatening.
2. Quantification of immunosuppression by flow cytometric assessment of intracellular cytokines in immunosuppressed patients gives more accurate information on the level of immunosuppression and this information can be used to improve monitoring of therapy.

OBJECTIVES

1. To prospectively survey eye disease in patients on immunosuppression for the prevention of rejection of heart, lung and heart-lung transplants.
2. To assess the value of screening in asymptomatic recipients of heart, lung and heart-lung transplants.
3. To investigate IL-2 and IFNγ production by T cells in the peripheral blood of:
    - Normal Subjects
    - Patients with autoimmune uveitis
    - Patients with HIV
4. To investigate the relationship between cytokine profiles and clinical profiles.
5. To assess if cytokine measurements could be a useful adjunct to the management of immunosuppressed patients.

LAYOUT OF THESIS

This thesis is divided into 5 chapters as follows:

Chapter 1 - Clinical Monitoring: Ocular Complications Following Heart, Lung and Heart-Lung Transplantation

Introduction, Aims, Methods, Results, Summary, Discussion

Chapter 2 - Quantification of Immunosuppression by Flow Cytometric Assessment of Intracellular Cytokines – A General Introduction

Introduction, Laboratory Techniques

Chapter 3 - Assessment of Cytokine Production in Autoimmune Uveitis Patients on Systemic Immunosuppression

Introduction, Aims, Methods, Results, Summary, Discussion

Chapter 4 - Assessment of Cytokine Production in HIV Positive Patients

Introduction, Aims, Methods, Results, Summary, Discussion

Chapter 5 – Overall Conclusions and Suggestions for Further Research

CHAPTER 1

CLINICAL MONITORING: OCULAR COMPLICATIONS FOLLOWING HEART, LUNG AND HEART-LUNG TRANSPLANTATION

1.1 INTRODUCTION

The traditional method of monitoring for toxicity is to monitor end-organ effects. As yet there are no laboratory indices that give a direct indication of end-organ toxicity in the eye so disease is picked up only when sufficient to cause ocular symptoms or by screening. Transplantation continues to be the arena in which immunosuppressive drugs are used in the largest quantities and at the highest doses and a myriad of ocular complications have been reported.

Heart, lung and heart-lung transplantations have become standard therapy for selected end-stage diseases. Advances in surgical and medical management and advances in immunosuppressive therapy have led to an improved quality of life and increased survival for transplant recipients. Allograft rejection is a constant risk, however, and the high levels of immunosuppression required to maintain the graft continues to place the recipient at risk of significant morbidity and mortality, particularly from infection. Ocular complications may affect quality of life after recovery and as survival improves it becomes increasingly important to identify and appropriately treat these conditions. The ocular complications that have been associated with transplantation can be grouped as follows:

- Opportunistic Infections
- Other Complications Associated with Infection
- Noninfectious Complications Secondary to Drug Toxicity
- Noninfectious Complications Associated with Underlying Disease Processes

1.1.1 Opportunistic Infections

Infections account for 18-20% of all mortality in transplant recipients(1). The risk of opportunistic infection is determined by the interaction between the patient's net state of immunosuppression and the epidemiological exposures the patient encounters (2). The patient's net state of immunosuppression is determined by a variety of pre and post-transplantation factors. The major post-transplantation factors are the nature of the intensive immunosuppressive therapy that is administered to maintain the graft and whether or not infection with one or more immunomodulating viruses such as cytomegalovirus and Epstein-Barr virus is present. Nosocomial factors documented to increase the risk of infection in the immunocompromised patient include central venous

catheterization, bladder catheterization, duration and intensity of systemic antibiotics, colonization, total parenteral nutrition, granulocytopenia, diabetic ketoacidosis, hospital construction and faulty air filtering mechanisms. Patients who become donors of solid organs are hospitalized before death and if critically ill, requiring intensive support such as ventilation, invasive cardiac monitoring and broad spectrum antibiotics, they are highly susceptible to the acquisition of nosocomial pathogens (3) (Figure 1.1). Lung transplant recipients are at highest risk for pulmonary infections as the transplanted organ has continuous contact with the extracorporeal environment(4). Different infections are characteristic at different points along the time course following transplantation and have been described as occurring in three phases:

1. early (less than 1 month);
2. intermediate (1-6 months);
3. late (greater than 6 months)(2, 5)

Generally speaking, most infections during the first month after transplantation are related to surgical complications and the anatomical region of the transplant dictates to a great extent the type of infection that occurs(6). The period following this is when most opportunistic infections tend to occur (Figure 1.2) and from 6 months most transplant recipients suffer from the same infections seen in the general community. Patients who have had frequent acute or chronic rejection are maintained on higher levels of immunosuppression for longer and so remain at risk of opportunistic infections for longer.

Because infection may be allograft and/or life threatening it is managed with a high index of suspicion and an arsenal of prophylaxis, preemptive and therapeutic treatment(2). Solid organ transplantation is a risk factor for endogenous endophthalmitis(7) (Figure 1.3) and a number of the infections characteristic of transplantation have typical ocular manifestations. An advantage of ocular examination is that accurate ocular diagnosis may sometimes offer clues to as yet undiagnosed co-existing systemic infections.

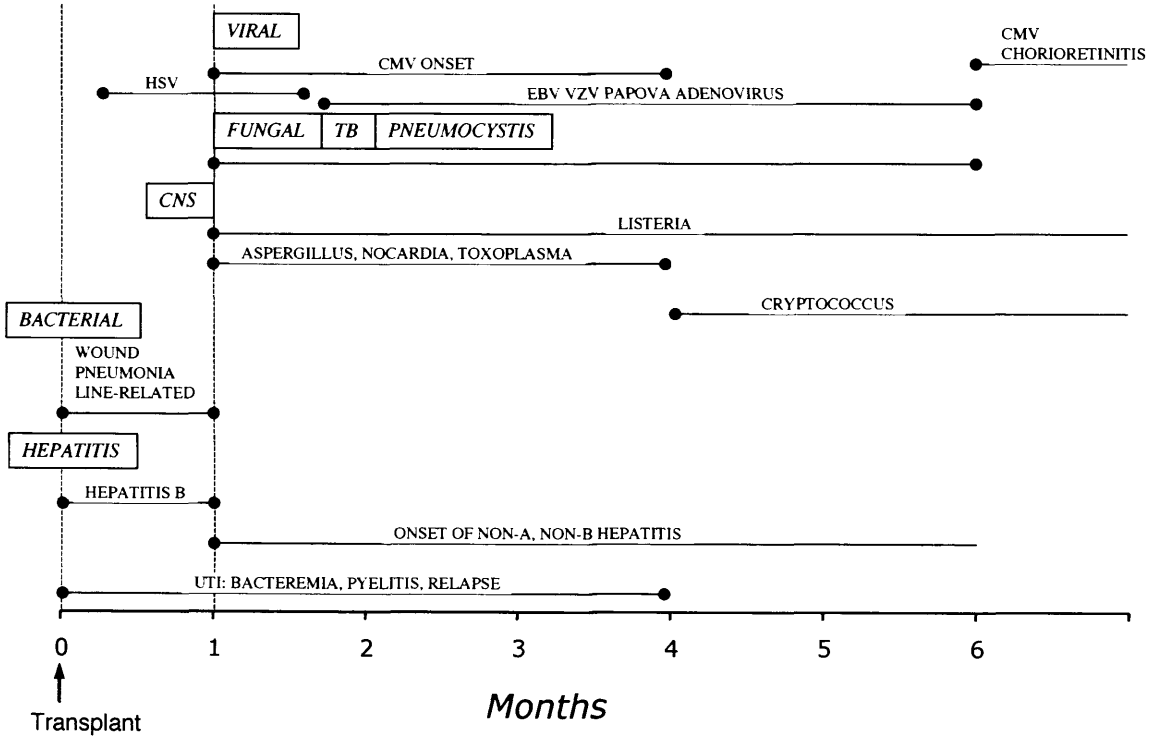
Figure 1.1: Incidence of Infectious Disease in Solid-Organ Transplant Recipients

Adapted from Snydman 2001[6]

	Incidence of infection (%) in patients receiving	
	Heart	**Lung/Heart-lung**
Bacterial	21-30	35-66
Cytomegalovirus	9-35	53-75
Herpes Simplex Virus	1-42	10-18
Varicella Zoster Virus	1-12	8-15
Candida spp	1-5	10-16
Mycelial Fungi	3-6	3-19
Pneumocystis jerovici	1-8	15

Figure 1.2: Timetable for the Occurrence of Infection After Transplantation

Adapted from Rubin et al. 1981[5]



1.1.1.1 Necrotizing Herpetic Retinopathies

Herpes viruses are the most common cause of viral infections in transplant recipients(1) and herpes group viral retinitis are the most common ocular opportunistic infections in solid organ transplant recipients (8).

1.1.1.1.1 Cytomegalovirus (CMV)

Human cytomegalovirus (Herpes virus type 5) is a double-stranded DNA virus that belongs to the β subgroup of herpes viruses(9). Infection leads to life-long persistence in 50-90% of the population and is generally subclinical in healthy subjects due to cellular and humoral immunity. In immunocompromised patients infection may lead to serious complications and CMV is responsible for a substantial fraction of the morbidity and mortality that follows organ transplantation (10, 11). Before prophylaxis was widely used the incidence of symptomatic CMV infection was 25% in heart transplant recipients and 39% in heart-lung transplant recipients(12). Active CMV in transplant recipients carries a mortality rate of about 5%(13). Infection may have 3 important consequences:

1. CMV disease,
2. immunomodulation leading to superinfection with opportunistic pathogens such as *Pneumocystis jerovici* and *Aspergillus fumigatus*,
3. injury to the transplanted organ(14).

The two main risk factors for developing symptomatic CMV disease are:

1. whether the donor, recipient or transfusion products are infected with latent virus capable of being reactivated following transplantation.
2. the nature of the immunosuppression administered following transplantation.

The clinical manifestations of CMV infection are exacerbated by increased immunosuppression, particularly by the use of antilymphocyte antibodies for induction therapy(14).

1.1.1.1.1.1 Clinical Features

The CMV syndrome in transplant patients occurs primarily in the first 3 months post-transplant when immunosuppression is at its most intense. It usually begins insidiously with fever and symptoms of anorexia, malaise, epigastric pain, myalgias and arthralgias. Approximately one third of those with constitutional symptoms develop a dry

nonproductive cough within the first week of onset and in lung and heart-lung transplant recipients CMV pneumonitis may be acute, progressing over a short period of time (hours to days) to lung white-out and respiratory failure. Myocarditis occurs rarely in heart transplant recipients.

CMV retinitis is an uncommon and late manifestation of CMV disease in transplant recipients usually occurring more that 6 months after transplantation. Retinitis typically presents as blurred vision. Fundal examination reveals characteristic focal, white areas of retinal necrosis with associated haemorrhage, perivascular infiltrates and minimal vitreous inflammation. Granular borders are characteristic. The natural history of untreated disease is one of progression to confluent involvement and healing with scarring and retinal detachment(15). Optic disc neovascularization has been described in the healing phase of CMV retinitis associated with renal transplantation(16).

There is a single report in the literature of a dendritiform epithelial keratitis in a cardiac transplant patient where histopathology and culture of corneal scrapings where highly suggestive of CMV epithelial keratitis(17). CMV disease affecting the cornea has more commonly been reported as endothelitis and conjunctivitis in patients with HIV(18, 19).

1.1.1.1.1.2 Prevalence of CMV Retinitis

The prevalence of CMV retinitis after solid organ transplantation is not known although before the onset of the AIDS pandemic immunosuppression associated with transplantation was responsible for most cases. Fishburne and colleagues (20) reviewed the records of 82 patients who had undergone cardiac transplantation between November 1986 and November 1994 (before CMV status matching and prophylaxis were routine) to attempt to determine the incidence of CMV retinitis after cardiac transplantation. Six out of 41 of the patients who had ocular examinations were considered to have evidence of CMV retinitis. Of these 6, only 2 had active infection and these were asymptomatic. Four of the patients were diagnosed after the infection had healed with characteristic scars. Two patients with healed CMV retinitis were symptomatic: one due to a retinal break at the border of the scarred retina and one due to cystoid macular oedema which resolved without treatment. All patients retained good visual function. They estimate the prevalence of CMV retinitis in cardiac transplant recipients to be 7.3% (6/82) and recommend screening of patients 3 to 4 months after transplantation and thereafter yearly or as symptoms occur. Erakgun (21) and colleagues assessed 13 asymptomatic cardiac transplantation patients in the post-

transplant period. They found that 15% (2 patients) had active CMV retinitis despite having no symptoms. These patients had histories of diabetes mellitus, arterial hypertension and heavy smoking and one patient had hyperlipidemia. They postulate that microvascular disease is an important risk factor for migration of CMV infected monocytes through retinal vessels. This hypothesis is supported by the fact that CMV retinitis is common in AIDS where microvasculopathy is characterisitic. They suggest screening patients with systemic microvascular risk factors such as diabetes, hypertension and smoking for the development of CMV retinitis after cardiac transplantation even if the patients have no visual symptoms.

Because of the important consequences of CMV infection large efforts are put into assessing risk factors for CMV disease, rapid detection of CMV in clinical specimens and the use of antiviral chemotherapy and immunoglobulin to prevent and treat CMV disease in the post transplant recipient.

1.1.1.1.1.3 Diagnosis

Although the ocular appearance is characteristic the diagnosis of systemic CMV is made difficult by the fact that a majority of transplant recipients have asymptomatic infection (with virus in saliva, urine and bronchoalveolar fluid) and only a minority will have symptomatic disease due to CMV. Ocular disease is often asymptomatic if the macula is not involved and therefore only uncovered on ocular examination(15, 21, 22). As organ rejection is the major differential diagnosis of CMV disease rapid diagnostic tests are used to inform the decision on whether to increase or decrease immunosuppression. These include virological tests such as the pp65 antigenemia assay and polymerase chain reaction (PCR) for DNAemia and, less commonly, serological tests such as complement fixing assay for CMV antibodies and ELISA. Diagnosis of ocular disease is often clinical and can usually be confirmed by PCR of vitreous samples(23).

1.1.1.1.1.4 Antiviral Strategies

The use of CMV seronegative blood products is effective in preventing CMV infection in CMV seronegative solid organ transplant recipients but these are scarce due to the high prevalence of CMV antibodies(12). Instead filtered or leukocyte-poor blood products which are associated with a very low risk of CMV transmission can be used for seronegative recipients(12). Protective matching of seronegative donors with

seronegative recipients is not possible due to the scarcity of donor organs but knowledge of CMV status for donor and recipient allows for risk stratification.

The options for prophylaxis against CMV are anti-CMV hyperimmune globulin, high-dose oral aciclovir or valaciclovir and IV ganciclovir +/- oral valganciclovir. Ganciclovir prophylaxis has been shown to be superior to aciclovir in heart and lung transplantation(13). Ganciclovir targets the viral DNA polymerase where it acts as a chain terminator: ganciclovir monophosphate is incorporated at the 3'end of the viral DNA chain following intracellular phosphorylation. The first phosphorylation step is catalyzed by CMV-encoded protein kinase(24). Valganciclovir is an oral prodrug of ganciclovir. Vaccination of seronegative renal transplant recipients with live, attenuated CMV has been shown to result in a more benign clinical course of disease although the incidence of primary infection was not affected(25). A recent meta-analysis of randomised controlled trials of prophylaxis with antiviral medications demonstrated a significant reduction in cytomegalovirus infection (1786 patients; relative risk 0.42 [95% CI 0.34-0.52]), cytomegalovirus disease (1300 patients; 0.26 [0.08-0.78]) and all-cause mortality in recipients of solid-organ transplants(26). Ganciclovir was seen to be more effective than acyclovir, and valganciclovir and intravenous ganciclovir were as effective as oral ganciclovir.

Pre-emptive therapy involves the administration of antiviral agents to a subgroup of patients prior to the appearance of disease. When successful it has advantages in terms of cost, emergence of resistant viral strains and medication side effects(12, 27). Pre-emptive therapy has been shown to reduce the incidence of CMV disease in heart or lung transplant recipients(10, 27). Ganciclovir treatment for CMV is given to those patients in whom surveillance has shown pre-symptomatic viremia 2 to 7 days before the onset of clinical symptoms consistent with CMV. Patients in whom immunosuppression is being augmented by either anti-lymphocyte antibody or pulsed steroids also benefit. Side effects include bone marrow toxicity, diarrhoea, renal toxicity and seizures. Although CMV antigenemia is generally considered to be a reliable and highly sensitive assay in cases of systemic CMV disease, this may not to be the case in isolated CMV retinitis occurring in transplant recipients(28). Also in contrast to other CMV diseases such as pneumonitis or colitis viral CMV load during retinitis does not necessarily correlate with disease progression(9).

Treatment with ganciclovir is indicated for all those who have evidence of invasive CMV disease. Treatment of ocular disease may require local therapy in addition to

systemic therapy. Either ganciclovir or foscarnet can be given by repeated intravitreal injection. Foscarnet is not frequently used systemically because of concerns about its significant renal toxicity in cyclosporine treated patients. It is a pyrophosphate analogue which interferes with the binding of the pyrophosphate to viral DNA polymerase during the DNA polymerization process(24). A ganciclovir sustained release implant (Vitrasert containing 4.5mg of ganciclovir) is also available for longer term control. Complications of intravitreal injection and vitrasert insertion are uncommon but include endophthalmitis, vitreous haemorrhage and retinal detachment. Fomivirsen (Vitravene) is the first antisense oligonucleotide analogue to be marketed. It is a 21-nucleotide sequence complementary to the immediate early region 2 of CMV messenger RNA. Intravitreal injection has been shown to be effective treatment of CMV retinitis in AIDS patients(29). Transient ocular complications include anterior chamber inflammation and raised intraocular pressure. Treatment with anti-CMV agents suppresses viral replication but does not eliminate the virus(30). Relapse is often not a problem in transplant patients as improvement in the recipient's immune function over time is likely and may even lead to regression and scarring without ocular treatment(15, 20-22).

CMV resistance to ganciclovir after prolonged treatment for CMV retinitis has been recognized amongst patients with AIDS since the late 1980s(31). Ganciclovir resistant CMV is only recently becoming a problem in solid organ transplant recipients. Likely causes of this are the widespread use of oral valganciclovir and the use of more intensive immunosuppressive strategies. Lung transplant recipients are at particular risk. Adding foscarnet is the intervention of choice for ganciclovir resistant CMV as cidofovir has a theoretical risk for cross resistance(31). CMV hyperimmune globulin may also be helpful.

1.1.1.1.2 Varicella Zoster Virus (VZV)

VZV (herpes virus type 3) belongs to the α subgroup of herpes viruses. Infection is almost universal with 95% of adults being VZV seropositive. The small proportion of recipients who are seronegative pre-transplantation are at risk of primary infection.

1.1.1.1.2.1 Clinical Features

Reactivation in those that are seropositive pre-transplantation will result in the characteristic localized vesicular eruption of herpes zoster. In one study the herpes zoster incidence was 15.1% after lung transplantation and 16.8% after heart

transplantation(32). The majority of recipients developed zoster during the first post-transplant year. Primary VZV in the solid organ transplant recipient can occur at any time after transplantation and although rare can cause a life threatening disseminated infection characterized by haemorrhagic pneumonia, skin lesions, encephalitis, pancreatitis, disseminated intravascular coagulation and hepatitis(11). It may also cause chicken pox or hepatitis alone.

1.1.1.1.2.2 Ocular Complications

In the general population the ophthalmic division of the trigeminal nerve is involved in 8-56% of herpes zoster cases and ocular complications develop in 50-71% of these(33). In a series of 11 patients who developed herpes zoster after cardiac transplantation one patient (9%) had ophthalmic involvement(34). Herpes zoster ophthalmicus has a multitude of manifestations including corneal hypoaesthesia, punctate keratopathy, pseudodendritic keratitis, stromal keratitis, neurotrophic keratitis, anterior uveitis and glaucoma(35). Posterior segment complications are less common.

Acute retinal necrosis syndrome (ARN) was reported first in otherwise healthy individuals but has been widely reported in immunosuppressed patients, particularly those with AIDS(33). Ng and colleagues reported 5 patients who developed ARN following heart, lung or liver transplantation(8). Diagnostic investigations were not performed so the herpes virus responsible is not known. ARN may be caused by a number of the herpes viruses and may be unilateral or bilateral. Clinically there is peripheral retinitis, which may be confluent, severe vitritis, retinal vasculitis and papillitis(36). Progression is rapid with early retinal detachment. Laser retinopexy has been used to prevent retinal detachment and there is usually a good response to high dose intravenous aciclovir.

VZV retinitis (previously known as progressive outer retinal necrosis (PORN) syndrome) was seen first in HIV patients. It is characterized by multi-focal deep posterior pole or peripheral retinal lesions with minimal, or no, intraocular inflammation. VZV retinitis progresses rapidly and bilaterality is common(37). It does not respond well to antiviral agents and is more likely to result in a poor visual outcome despite treatment with multiple agents(38). Retinal detachment is likely. There are scattered reports in the literature of VZV retinitis in patients with other causes for immunosuppression including bone marrow and renal transplantation(39-42) and in

immunocompetent patients (43). VZV retinitis was not seen in the Ng series of ocular complications following heart, lung and liver transplantation(8).

1.1.1.1.2.3 Diagnosis

Diagnosis of VZV is usually made on clinical appearance. Standard criteria exist for the diagnosis of ARN(36). Clinical suspicions are best confirmed by PCR for VZV DNA and/or the Goldmann Witmer coefficient to determine levels of local antibody production. PCR may also be used to exclude CMV. Culture of virus in susceptible culture lines, demonstration of multinucleated giant cells on Tzanck smear and/or immunofluorescence techniques may also be helpful but these tests are rarely used clinically.

1.1.1.1.2.4 Treatment

Primary VZV has a high mortality in this group of patients so seronegative patients are advised to promptly report all exposures to VZV so that varicella-zoster immune globulin can be administered within 72hrs of exposure. Receiving prolonged CMV prophylaxis does not decrease the risk of developing herpes zoster although it does reduce the mortality(32). Live attenuated VZV vaccine given several months prior to transplantation is increasingly used particularly in the paediatric and seronegative transplant populations(44, 45). Aciclovir targets the viral DNA polymerase acting as a chain terminator, following intracellular phosphorylation. The first phosphorylation step is catalyzed by the virus encoded thymidine kinase making aciclovir specific for VZV (and HSV1 and 2)(24). IV aciclovir is administered if a zoster skin rash occurs post-transplantation but despite these measures progression to dissemination and death can still occur. Famciclovir or valaciclovir may also be used. Oral valganciclovir has been used successfully for the treatment of ARN in an immunocompetent patient(46).

1.1.1.1.3 Herpes Simplex Virus (HSV)

HSV (herpes virus type 1 and 2) belong to the α subgroup of herpes viruses. Reactivated HSV is the most common viral infection during the first month after transplantation(6). 75% of adult solid organ transplant recipients have HSV antibodies(11). Primary infection is usually a childhood illness and is often subclinical. Primary infection post-transplantation is very rare and may be transmitted by person-to-person contact or via the allograft. Both HSV1 and 2 may affect the eye.

1.1.1.1.3.1 Clinical Features

Following primary infection the virus remains latent in the sensory nerve ganglia until reactivation leads to oral, corneal or genital lesions usually in the first month post-transplantation in about one third of adult transplant recipients(11). Reactivation or primary HSV occasionally causes pneumonitis, tracheobronchitis, oesophagitis, hepatitis and rarely disseminated infection.

Typical ophthalmic features are lid and conjunctival vesicles, dendritic epithelial corneal ulcers, stromal keratitis and anterior uveitis(47). HSV may affect the retina producing ARN syndrome, especially in younger patients(47, 48).

1.1.1.1.3.2 Diagnosis

The diagnosis is usually made clinically and can be confirmed by laboratory techniques. A positive IgM titer or a fourfold or greater rise in IgG titer are diagnostic. PCR of vitreous samples is useful for the diagnosis of ARN and to exclude CMV.

1.1.1.1.3.3 Treatment

The prophylactic use of oral aciclovir in the immediate post-transplant period has significantly reduced the incidence of HSV (6). Treatment of HSV infection including ocular infection is with aciclovir, the dose and route of therapy depending on the site and severity of infection. Chorioretinal disease responds to systemic treatment. Side effects of aciclovir infusion include phlebitis after IV infusion, renal toxicity and rash. HSV pneumonia is associated with a mortality rate of up to 75% despite treatment with aciclovir in solid-organ transplant recipients(11). Despite widespread aciclovir use HSV resistance has not been a problem in solid organ transplant recipients.

1.1.1.2 Fungal Chorioretinopathies

Fungal infections are reported in all types of solid organ transplantation and are associated with higher mortality than other forms of infection (11, 49). They are generally rare in the first month after transplantation (see Figure 1.2) (3, 5). Nosocomial fungal infections (primarily owing to *Candida, Aspergillus and fusarium* spp) usually occur within the first 2 months after transplantation, at a time that correlates with the most intense immunosuppression. Late rejection requiring increased immunosuppression contributes to delayed onset of cryptococosis, *Pneumocystis jerovici* and the endemic mycoses. Fungal endophthalmitis is most likely in the setting of fungal bronchopneumonia in a transplanted lung. Drug resistance leads to an abysmal outcome and drug resistant bilateral endogenous *Scedosporium prolificans*

endophthalmitis with a fatal outcome has been reported following lung transplantation(50).

1.1.1.2.1 Aspergillus spp.

Aspergillus fumigatus, A.flavus, A.niger and A.terreus are the most common mycelial fungi to cause nosocomial infection in solid-organ transplant recipients(11).

1.1.1.2.1.1 Clinical Features

The site of primary infection is usually the lungs (51, 52) and disseminated aspergillosis occurs via haematogenous spread. Heart, lung and heart-lung transplantation are associated with a higher rate of pulmonary aspergillosis than other solid organ transplants(3). In one study, 4.5% of heart transplant recipients seen over a 9 year period developed invasive aspergillosis(53) and in another, 15% of all heart-lung transplant recipients developed *Aspergillus* infection (4).

Alveolar macrophages normally kill inhaled aspergillal conidia, while functioning neutrophils eliminate residual mycelia(11). Neutrophil and macrophage dysfunction present in solid organ transplant recipients treated with steroids favour the development of aspergillosis. Once tissue infection develops, invasion of the blood vessels is the rule with tissue infarction, haemorrhage and dissemination with metastatic seeding. Most *Aspergillus spp* infections occur within the first 3 months following solid organ transplantion(5)(see Figure 1.2). Disseminated aspergillosis is usually fatal despite intensive treatment(11, 51, 54-56).

Endogenous *Aspergillus* endophthalmitis is a rare manifestation of disseminated aspergillosis and often occurs in conjunction with endocarditis(11). A literature review of 86 patients with endogenous proven *Aspergillus* endophthalmitis since 1949 revealed that 23% of the patients had undergone a solid organ transplant and 17% of the 86 patients had lung disease (57). The typical appearance is of grey-white choroidal masses with adjacent retinitis, haemorrhage and fluffy collections in the vitreous. A "pseudohypopyon" or preretinal layering of exudate has also been described(57). There may also be conjunctival injection, chemosis, anterior chamber activity and hypopyon. *Aspergillus* endophthalmitis is usually rapidly progressive.

1.1.1.2.1.2 Diagnosis

Diagnosis of systemic disease is made difficult by the fact that the isolation of *Aspergillus spp.* from respiratory and wound specimens does not always imply disease as the fungus may be a colonizer or a laboratory contaminant. Helmi et al reported that

53% of their lung transplant recipients were colonized with *Aspergillus spp.* prior to transplantation(58). Ensuring that solid organ transplant recipients are not cared for close to building works and that flowers and potted plants are not allowed in patient's rooms reduces colonization rates. Infection is certain if fungal filaments are present. Early recognition of the ocular disease may aid in diagnosis and prompt systemic treatment.

Pathological specimens demonstrate numerous branching and septate hyphae in thrombosed choroidal vessels (51, 54, 55). Diagnosis is often delayed with 51% of patients in the survey by Riddell and colleagues having the diagnosis made after enucleation or at autopsy (57). The diagnosis was made by culture or fungal stain in 90% of vitrectomy specimens taken and 50% of vitreous aspirates. Blood culture and anterior chamber samples were rarely revealing.

1.1.1.2.1.3 Treatment

Amphotericin B is a polyene antifungal that was first isolated from *Streptomyces spp.* in 1956 (59). It is poorly soluble in water and binds to sterols in fungal cell membranes leading to disruption of the osmotic integrity of the membrane and cellular death. The range of activity is very broad and it remains the mainstay of therapy for most life threatening fungal infections in solid organ transplant recipients(3). Side effects of treatment include fevers and myalgias which occur commonly and nephrotoxicity and bone marrow toxicity which are less frequent. Nephrotoxicity may limit the use of amphotericin B particularly in patients on CsA(3). Formulations with lipid carriers such as liposomal amphotericin B have improved toxicity profiles. *A. terreus* is notably resistant to amphotericin B but is sensitive to voriconazole (60). Voriconazole is a second generation triazole agent based on fluconazole that has been shown to be safe and effective in the treatment of invasive mould infections in transplant recipients(61, 62). It does, however, increase the levels of immunosuppressive drugs such as tacrolimus and CsA on coadministration so care must be taken to avoid renal toxicity(62). Azole antifungals inhibit fungal cytochrome P-450 enzymes resulting in faulty ergosterol synthesis and production of a defective cell membrane. Voriconazole can be administered intravenously if required and treatment can be continued orally. It is well tolerated and the main adverse effects appear to be liver disturbance and mild and transient visual disturbance such as blurred vision, altered visual perception and photophobia. These effects are not sight-threatening and do not necessitate a change in therapy.

Amphotericin B has been the drug of first choice for the treatment of *Aspergillus* endophthalmitis but due to the extensive vascular occlusion penetration is poor and early diagnostic pars plana vitrectomy with empirical intravitreal amphotericin B is recommended to deliver adequate drug levels to the eye(51, 57, 63). The half life of amphotericin B in the vitrectomized eye is only 2 days (64) so repeated injections are required. When intravitreal injections are given systemic treatment must also be given to control the systemic disease. Fluconazole and itraconazole cannot be used to treat ocular *Aspergillus* infection because of their poor penetration into the eye but oral voriconazole has been shown to achieve broad spectrum fungicidal concentrations in the vitreous of uninflamed eyes and may prove to be a useful adjunct to treatment(65). Aliyeva and colleagues have reported a case of Aspergillus endophthalmitis following cardiac transplantation that was treated with oral voriconazole(66). Although this patient proceeded to enucleation the fellow eye remained unaffected despite systemic disease and histology showed fungal elements restricted to the preretinal inflammatory infiltrate and notably absent from the choroidal and retinal vessels.

Despite intensive therapy progression to severe visual impairment, enucleation and/or death remain likely (7, 57). In one study disseminated aspergillosis was the single infectious complication responsible for the highest mortality rates in a heart transplantation population(67). Voriconazole for systemic disease has been associated with a higher survival rate than amphotericin B followed by other antifungal agents(61). Of the 86 patients with *Aspergillus* endophthalmitis in Riddell's review only 9 (10%) regained useful vision and 2 of these had very early disease restricted to the anterior segment(57).

1.1.1.2.2 Candida spp

The yeast *Candida albicans* is the most common agent in systemic and ocular infection although *C.kruseii, C. glabrata, C. zeylanoides* and *C.tropicalis* are also reported(11). Cell-mediated immunity, macrophage and neutrophil function are the defense against candidal infections. Cell mediated immunity is compromised by immunosuppressive therapy and many other factors in the solid organ transplant recipient such as antibiotic use, diabetes mellitus and indwelling bladder and intravenous catheters lead to increased candidal colonization(11). Candidemia leads to disseminated disease in 50% of transplant recipients affected and is associated with increased mortality rates(3).

1.1.1.2.2.1 Clinical Features

Infection can present in a myriad of ways including catheter-related sepsis, pulmonary infection, urinary tract infection, arthritis, endocarditis, aortitis, brain abscess, mediastinitis and endophthalmitis. Candidal infection in heart and heart-lung transplant recipients may be a cause of sudden death either due to rupture of the aortic anastomosis secondary to mycotic aneurysms or due to mediastinal abscesses secondary to dehiscence of an airway anastomosis(11).

Blood borne *Candida* reaches the choroid, forms collar-stud abcesses into the retina and thus enters the vitreous giving a characteristic clinical appearance. Small, multiple abscesses are seen with foci of retinal damage and vitreous snowballs(68, 69). The endophthalmitis has a relatively indolent course with better treatment results than for *Aspergillus* endophthalmitis(7, 70, 71). Rao and colleagues performed a retrospective study of 25 patients who underwent enucleation, 13 with *Aspergillus* disease and 12 with *Candida* disease. They identified 4 cases of endogenous endophthalmitis due to *Aspergillus spp.* in solid organ transplant recipients. None of their patients with *Candida* endophthalmitis had had an organ transplant. They propose that drug induced immune suppression and organ transplantation are significant risk factors for aspergillosis and less so for *Candida* endophthalmitis (68, 71).

1.1.1.2.2.2 Diagnosis

Diagnosis of infection is by obtaining yeast fungal stains and cultures of appropriate specimens. In cases of endophthalmitis vitreous samples can be obtained at vitrectomy.

1.1.1.2.2.3 Treatment

A North American prospective observational study assessed ocular candidiasis in 118 patients with candidemia(72). None of the patients in this study developed endophthalmitis. Only 11 patients (9%) developed chorioretinitis and this was effectively treated by prompt systemic treatment initiated for the fungemia. They found that the presence of visual symptoms and immunosuppression were significant risk factors for ocular involvement and conclude that Candida endophthalmitis is likely to represent chorioretinal extension in patients with delayed antifungal therapy(70, 72). Donahue and colleagues propose ophthalmology examinations in patients with candidemia to allow an objective measure of therapeutic response and so that more aggressive local therapy can be initiated in cases of progression to endophthalmitis. Amphotericin B is the mainstay of treatment. Because of the high incidence of nephrotoxicity with amphotericin B azole antifungals such as fluconazole or

itraconazole may be useful for systemic candidemia if sensitivity is demonstrated. Voriconazole has shown potent in vitro activity against *Candida* spp including *C. krusei* and less susceptible isolates(62). Ketoconazole is not used because of low efficacy, liver toxicity, interaction with CsA and erratic gastrointestinal absorption.

Treatment of severe ocular disease is with vitrectomy and intravitreal amphotericin B, while milder disease can be treated with intravitreal amphotericin B without vitrectomy. 76-100% of patients are reported to show recovery of vision(70). Systemic treatment is also required and early treatment of candidemia may reduce the incidence of endophthalmitis.

1.1.1.2.3 Cryptococcus neoformans

Cryptococcus is an encapsulated yeast. The species *C.neoformans* is the only one to be pathogenic in man. It has a large polysaccharide capsule with antiphagocytic properties and this together with its ability to grow at 37°C and the enzyme phenol oxidase make it a successful pathogen. It is widespread and found in soil, especially soil containing bird droppings. Opportunistic infection is most likely to occur later in the time course following transplantation (5)(see Figure 1.2).

1.1.1.2.3.1 Clinical Features

Infection is by inhalation and the primary lesion is usually in the lung and may be asymptomatic but followed by haematogenous dissemination to other areas of the body. Manifestations include meningitis, pneumonia, pleural infection, cutaneous lesions, arthritis, pyelonephritis and pyrexia of unknown origin. The organism has a predilection for the CNS. Ocular involvement is usually a secondary consequence of central nervous system involvement. Approximately 40% of individuals with cryptococcal meningitis have eye related signs such as papilloedema, optic atrophy or extraocular muscle paresis(73).

Endogenous ocular cryptococcosis is rare and is primarily a multifocal choroidal disease with secondary invasion of the retina and other intraocular structures. The vitreous overlying the chorioretinal lesions usually remains clear (73, 74). Crump and colleagues reported 27 patients with cryptococcal endophthalmitis over a period of 23 years(75). Five (18%) of these patients were on immunosuppressive therapy although none of these patients was a transplant recipient. Litmathe and colleagues reported a patient with endophthalmitis which progressed to a scleral abscess following heart transplantation(73).

1.1.1.2.3.2 Diagnosis

Diagnosis of systemic disease is usually by the cryptococcal antigen test performed on serum and cerebrospinal fluid. This is also useful for monitoring the response to therapy. Culture and fungal stains are also helpful.

1.1.1.2.3.3 Treatment

Recommended treatment of systemic disease is with amphotericin B combined with flucytosine followed by an azole. Intravenous amphotericin B will not, however penetrate effectively into the eye. Flucytosine does penetrate the eye and is an antimetabolite that is activated by deamination within fungal cells to 5-fluorouracil (5-FU)(76). It thus inhibits fungal protein synthesis by replacing uracil with 5-FU in fungal RNA. Flucytosine also inhibits thymidylate synthetase and thus interferes with fungal DNA synthesis. It is used in combination to reduce the emergence of resistant strains. Gastrointestinal side-effects, bone marrow depression and hepatotoxicity can occur.

1.1.1.2.4 Pneumocystis jerovici (carinii)

Pneumocystis has recently been found to be host specific so the previously named *P.carinii* has been renamed *P.jerovici*. *P.carinii* is reserved for species pathogenic in rats. Although it has protozoa-like features and debate continues *Pneumocystis* has been reclassified as an *Archiascomycetous* fungus. *Pneumocystis* pneumonia occurs in approximately 3-10% of heart transplant recipients not given prophylaxis, often within the first 4 months after transplantation (11, 77, 78). *Pneumocystis* infection in the absence of effective prophylaxis occurs more commonly in lung and especially heart-lung transplant recipients than in other solid-organ groups. One prospective study of heart-lung transplant recipients using serial BAL specimens found *Pneumocystis* in 88% of the patients; infection was symptomatic in 35%, subclinical in 24%, and asymptomatic in 41% (79).

1.1.1.2.4.1 Clinical Features

Pneumocystis pneumonia in solid-organ transplant recipients typically has a subacute presentation, with fever, dyspnoea, non-productive cough, radiographic findings of interstitial infiltrates, and hypoxemia out of proportion to the physical findings. *P.jerovici* is frequently isolated with CMV(11, 78). Dissemination may occur via lymphatics or blood. It most commonly involves thyroid, liver, bone marrow, lymph nodes and spleen(80).

Pneumocystis choroidopathy was first described in AIDS patients(81). Clinically there are multifocal, bilateral, plaque-like yellow choroidal lesions which are one-third to two disc diameters in size in the posterior pole and mid-periphery. There is no vitritis. Histopathologically there are eosinophilic, cellular, vacuolated, frothy choroidal infiltrates (81). At the time of writing *Pneumocystis* choroidopathy has not been reported in solid organ transplant patients.

1.1.1.2.4.2 Diagnosis

The diagnosis is made by histological examination of bronchoalveolar lavage fluid or tissue biopsy. Immunocytochemistry and PCR are also helpful. *P. jerovici* cannot be readily cultured. The ocular features are highly characteristic, although not always symptomatic, and the initial diagnosis of disseminated *Pneumocystis* may be made by the ophthalmologist(81).

1.1.1.2.4.3 Treatment

Low-dose co-trimoxazole is relatively non-toxic and is very effective prophylaxis against *Pneumocystis* pneumonia (3, 82). It is advised for all solid-organ transplant recipients for at least 6 months post transplantation(11). Prophylaxis beyond 6 months post-transplantation is indicated for heart-lung and lung transplant recipients and for patients with ongoing risk factors for *Pneumocystis* pneumonia such as multiple episodes of rejection, treatment with OKT3 monoclonal antibodies or persistent allograft dysfunction. Low dose co-trimoxazole for a minimum of 6 months also provides efficient protection against urinary tract infections, listeria sepsis, nocardiosis and toxoplasmosis (82, 83). Alternatives to co-trimoxazole for *Pneumocystis* prophylaxis include aerosolized pentamidine, dapsone, clindamycin and primaquine and atovoquone(11). Aerosolized pentamidine does not provide adequate prophylaxis against *Pneumocystis* choroidopathy in AIDS patients(81, 84, 85).

Treatment is with high dose co-trimoxazole or, if this is not possible with intravenous pentamidine for 14 to 21 days. Systemic treatment is also effective for ocular disease. Adverse effects of co-trimoxazole include anorexia, nausea, vomiting, a transient rise in creatinine levels and hyponatremia; adverse effects of pentamidine include renal dysfunction, bone marrow depression, hypoglycemia, hypocalcemia, liver dysfunction, and seizures(11).

1.1.1.3 Other Chorioretinopathies

1.1.1.3.1 Toxoplasma gondii

Toxoplasma gondi is an obligate intracellular protozoan for whom the cat family is the definitive host and humans are an intermediate host. Infection with *T.gondii* is widespread and primary infection is usually asymptomatic with latency. *T.gondii* disease in solid organ transplant patients may occur secondary to reactivation or due to new infection from the donor organ, transfusion products or food such as uncooked meat and vegetables. Intracellular multiplication of tachyzoites leads to foci of necrosis. *T.gondii* is most commonly reported in heart transplantation because of the predilection of the parasite to invade muscle tissue. Gallino and colleagues found a prevalence of 13% after cardiac transplantation(86). Seronegative recipients receiving an organ from a seropositive donor are at the highest risk with 57% of heart transplants(87), 20% of liver and <1% of kidney transplant recipients acquiring primary *T. gondii* infection in the absence of prophylaxis(11, 88). In one study 25% of D+/R- heart transplant patients who were not taking prophylaxis developed lethal toxoplasmosis(67).

1.1.1.3.1.1 Clinical Features

Clinical presentations include meningoencephalitis, brain abscess, pneumonia, myocarditis, pericarditis, hepatitis, and choroidoretinitis(11). The onset of clinically significant infection has been reported to be between 1 day and 7 years after transplantation, although most cases occur within 2 months post-transplantation(11). Fulminant toxoplasmosis leading to death has been reported in heart transplant recipients(89, 90).

Ocular toxoplasmosis is reported after heart, lung or heart lung transplantation(86, 90-92). *T.gondii* choroidoretinitis was seen in 4 out of 175 patients who underwent cardiac transplantation at a single institution between 1989 and 2002(92). Three of the patients had acquired disease and one had disease reactivation. Diagnosis of *T. gondii* choroidoretinitis is generally made clinically in the immunocompetent patient but in immunocompromised patients diagnosis may be more difficult as inflammation may be minimal and the lesion may be more extensively necrotic or haemorrhagic than typical(91, 92). The typical pigmented scar from previous episode of inflammation may also be absent.

1.1.1.3.1.2 Diagnosis

Definitive diagnosis is made by the histological demonstration of tachyzoites with surrounding inflammation in tissue. This may be very difficult to demonstrate

especially in small samples. *T. gondii* infection is widespread so serological testing is often not very useful. The Sabin-Feldman dye test is the most specific test. A positive IgM titre or a fourfold rise in the IgG titer supports a diagnosis of toxoplasmosis(86). The Goldman-Witmer coefficient between vitreous and serum (11, 92) or between aqueous humor and serum(93) can also be used to assess infection. Comparison of antibody production with immunoblotting techniques is also helpful(93). *T.gondii* specific DNA can sometimes be demonstrated by PCR in affected tissue. Serological investigations and PCR for CMV may be useful to exclude CMV retinitis. Multiple infections are more likely in the immunosuppressed patient and concomitant CMV infection may occur(90).

1.1.1.3.1.3 Treatment

Prophylaxis is advised when a seronegative patient receives an organ from a seropositive donor(3, 87, 92, 94). The standard regime is co-trimoxazole as used for *Pneumocystis* prophylaxis administered for at least 6 months post transplantation(95). Daily pyrimethamine may also be used and has been shown to reduce the incidence of *T.gondii* infection from 57% to 14% in mismatched heart and heart-lung transplant recipients(94). Alimentary and hygiene precautions are as important in the seronegative organ recipient as in pregnancy in seronegative mothers(96), and patients are advised not to consume poorly cooked meat or soiled fruits or vegetables and to avoid contact with cats.

Standard treatment with pyrimethamine with folinic acid and sulfadiazine for 4-6 weeks is usually effective although primethamine may result in bone marrow toxicity and sulfadiazine in renal toxicity. Clindamycin is an alternative. Ocular disease responds well to systemic treatment.

1.1.1.3.2 Nocardia asteroides

N asteroides is a gram positive aerobic actinomycete bacterium with both bacillary and coccoid elements(97). It is soil-borne and often found in decaying organic matter such as wet hay or straw. Other disease causing species exist including *N. brasiliensis*. It has a worldwide distribution and can be recovered from the skin or upper respiratory tract of healthy people.

1.1.1.3.2.1 Clinical Features

Infection may be localized or disseminated and the lungs are the most common portal of entry although infection may occur through penetration of skin, mouth, intestines or the

eye. Up to 50% of nocardiosis occurs in otherwise healthy patients but it is common and has a worse prognosis in debilitated, immunocompromised or immunosuppressed patients(97). Infection is usually chronic and progressive. A series of heart transplant patients has shown a 10% incidence of systemic nocardiosis (98) and nosocomial outbreaks have been reported in heart transplant units (99). The disease becomes disseminated to the central nervous system in 23% of cases and ocular involvement has been reported in 3% of cases of systemic nocardiosis (100).

Intraocular nocardiosis has been reported following heart transplantation (101, 102) and following renal and bone marrow transplantation(97, 103, 104). The case reported by Chaudhry and colleagues had biopsy proven pulmonary nocardia and complained of blurry vision. The visual acuity was 20/20 on both sides but examination revealed yellow, nummular, choroidal abscesses with overlying exudative retinal detachment and minimal vitritis. The authors reported marked resolution following treatment with intravenous imipenem-cilastin. However, the patient immediately developed an area of retinitis and polymerase chain reaction in the blood was positive for CMV. This responded well to treatment with IV ganciclovir. The case illustrates the possibility of co-infections in immunosuppressed patients. Keratitis, conjunctivitis, corneal ulceration and episcleral granulomas are other reported manifestations of intraocular nocardiosis(104).

1.1.1.3.2.2 Diagnosis

Definitive diagnosis is by culture of the organism.

1.1.1.3.2.3 Treatment

Most strains of *N. asteroides* are sensitive to antimicrobial therapy. Amikacin is often the treatment of first choice (105). Sulfonomides in combination with trimethoprim are usually effective(11). Treatment must usually be prolonged as there is a tendency for relapse and prognosis depends upon the extent of the disease and the patients underlying immunologic status.

1.1.1.4 Other Bacterial Infections

Bacterial infections occur in 54% of lung transplant recipients and 21-30% of heart transplant recipients, usually within 2 months after transplantation (82). They most commonly involve the lungs, the urinary tract, surgical wounds or intravascular lines and are similar to those that occur in surgical patients who are not immunosuppressed. Perioperative antibacterial prophylaxis is standard practice in transplant centres.

Dissemination of bacterial infection including mycobacteria to the eye following transplantation appears to be rare. In a study of 620 consecutive patients undergoing orthotopic heart transplantation at Stanford Medical Centre there were 1073 infectious episodes(67). None of the bacterial infections involved the retina. A major review of endogenous bacterial endophthalmitis did not identify transplantation as a significant predisposing factor(106) although it was identified in a series of culture proven endogenous endophthalmitis including both fungal and bacterial causes(7) (Figure 1.3). A recent retrospective report of endogenous endophthalmitis in Australia found that 1 out of 13 patients was post transplant (renal) and this patient died from disseminated *A. fumigatus* infection(107). No patient who developed bacterial endophthalmitis had transplantation as a risk factor.

Figure 1.3: Systemic Medical Conditions Associated with Endogenous Endophthalmitis Adapted from Schiedler 2004[7]

Medical Condition	Number of Patients*
Intravenous line or indwelling catheter	14
Diabetes Mellitus**	11
Immunosuppressive Therapy	9
Organ Transplantation	6
Malignancy	5
Haemodialysis	5
Intravenous Drug Use	3
Human Immunodeficiency Virus	1

*21 patients. 14 patients had more than one associated condition.

**2 patients had steroid-induced hyperglycaemia

1.1.2 Other Complications Associated with Infection

1.1.2.1 Post Transplantation Lymphoproliferative Disorder (PTLD)

Another complication of organ transplantation related to immunosuppression is post transplantation lymphoproliferative disorder (PTLD). This is an abnormal proliferation of lymphocytes strongly associated with Epstein Barr Virus (EBV) infection (108). EBV is herpes virus type 4. CsA inhibits the virus-specific cytotoxic T cells that normally control the expression of EBV infected transformed B cells and this allows the development of B cell tumours. These range from benign polyclonal polymorphic B lymphocyte hyperplasia to monoclonal B lymphocyte lymphoma. 10% of PTLD is, however, of T cell origin suggesting that the condition may sometimes be independent of EBV(108).

The overall prevalence of PTLD is less than 5% (109) but rates vary with the type of organ transplanted, primary EBV infection, CMV mismatch and the type and intensity of the immunosuppression(11, 108, 110-113). It has been reported in 5 to 13% of heart transplant recipients and 9% of heart-lung transplant recipients(110). The incidence of PTLD is highest in the first post-transplant year(114) but for those with ocular involvement the latency is often years after transplantation(115, 116). Ocular involvement has been reported after heart transplantation(115, 117) and in a lung transplant recipient(118). It is most commonly reported in paediatric patients who have undergone liver transplantation(116, 119-124).

1.1.2.1.1 Clinical Features

EBV infection is usually asymptomatic or mild. Systemic manifestations of infection can take 3 forms: an infectious mononucleosis type illness, disseminated disease with a high mortality and focal organ system involvement(108). 50% of paediatric transplant recipients are seronegative for EBV and in one transplant centre symptomatic EBV infections occurred in 19% of children and 2.7% of adults(113).

Ocular manifestations were first described by Brodsky and colleagues as iris nodules, iris masses with a uveitic masquerade syndrome, vitritis, subretinal masses and chorioretinitis(124). Anterior chamber cells and iris nodules are the most common manifestations. Orbital involvement has also been reported(123). Demols and colleagues were able to demonstrate more than 1000 times the levels of EBV genomes by semiquantitive PCR in the vitreous of an affected patient compared to the

blood(125). The clinical course of disease is described as chronic and fluctuating(116) and corticosteroids are poorly effective. In approximately 50% of patients ocular PTLD is part of a systemic disease(108).

1.1.2.1.2 Diagnosis

Definitive diagnosis is by the histological finding of mono or polyclonal B or T cell proliferation.

1.1.2.1.3 Treatment

Therapy requires a reduction in immunosuppressants to balance the immune response. Antivirals are also given to control the EBV infection although it has been suggested that virus replication might not play as important a role in pathogenesis during the later stages of PTLD as it does at disease onset(116). The response to therapy is often slow and local therapy with radiation(117, 124) and surgical excision may also be required(122).

1.1.2.2 Kaposi's Sarcoma (KS)

Human herpes virus 8 (HHV8) appears to be central to the aetiology of Kaposi's Sarcoma (KS) and has been associated with KS in transplant patients. Post transplantation KS may be due to HHV8 reactivation as a result of immunosuppressive treatment or to HHV8 primary infection transmitted via organ transplantation(126). KS appears at a mean of 22 months post transplantation and is the malignancy appearing earliest following transplantation.

1.1.2.2.1 Clinical Features

Lesions are characterized by multiple red or violaceous plaques or nodules composed of a proliferation of vascular channels and spindle-shaped neoplastic cells. The most common site of involvement of KS is the skin and periocular skin and conjunctiva may be affected. 40% of transplant patients may also have visceral disease(127, 128). The immunosuppression associated with organ transplantation is a risk factor for KS and the incidence of KS among transplant recipients in North America is 3-4% compared to 0.02% among the non HIV infected population (127). It is rarely reported in heart(126, 128, 129) and lung(130) transplant patients. One patient in the series reported by Barete and colleagues had conjunctival involvement.

1.1.2.2.2 Diagnosis

Diagnosis is usually clinical and may be confirmed by the histological finding of proliferation of vascular channels and spindle-shaped neoplastic cells.

1.1.2.2.3 Treatment

KS is associated with calcineurin inhibitors, particularly CsA, and reduction of immunosuppression or conversion to immunosuppressives with a different mechanism of action, such as sirolimus, is usually effective treatment(131-139). Ocular KS may not require intervention(38) as the lesions are slow growing, do not invade the eye and often do not affect the vision. Eyelid or other organ involvement may require intralesional therapy, radiotherapy, reduction in immunosuppression, chemotherapy or surgery. When visceral involvement is present mortality may be as high as 60-75% despite treatment(128).

1.1.2.3 Progressive Multifocal Leukoencephalopathy (PML)

JC virus has been cultivated from human brain of PML patients(140). This virus belongs to the polyoma subgroup of the genus Papovaviridae and is ubiquitous (the prevalence of seropositivity ranges from 50% in children to 90% in adults)(141). Visual loss due to PML occurs in 50% of cases as there appears to be a predeliction for the occipital cortex. Visual loss has been reported as the presenting symptom of PML in a heart transplant recipient(142) and after lung transplantation(143).

1.1.2.3.1 Clinical Features

Primary infection usually occurs during childhood and is asymptomatic or causes fever. Recovery occurs with latency. Once reactivated the virus may penetrate the brain leading neurological sequelae.

1.1.2.3.2 Diagnosis

The diagnosis is made by demonstrating JC virus in the cerebrospinal fluid by PCR or by brain biopsy. Histologically there are multifocal sites of demyelination with sparing of axons, characteristic gigantic astrocytes with large pleomorphic nuclei and large hyperchromatic oligodendrocytes at the periphery of the lesion(142). Diagnosis is made difficult by the fact that JC virus may not be demonstrated in CSF until months after the initial presentation(143).

1.1.2.3.3 Treatment

Untreated mortality is very high but there has been success with combinations of cytarabine and other antivirals including ART in the HIV infected population(143).

1.1.3 Noninfectious Ocular Complications Secondary to Drug Toxicity

There are difficulties is ascribing particular toxic effects to particular drugs when transplant recipients are exposed to a cocktail of drugs at varying doses for extended periods of time. Despite this the drugs used in transplantation characteristically produce a number of ocular side effects as described below.

1.1.3.1 Corticosteroids

1.1.3.1.1 Posterior Subcapsular Lens Opacity and Cataract

Posterior subcapsular lens opacities (PSCLO) are a well recognized and studied complication of long-term oral corticosteroid use(144-148) and the incidence of cataract appears to rise with higher dosage and longer duration of corticosteroid therapy. The mechanisms involved in cataract development are not clear but probably include increased glucose levels, caused by an increased gluconeogenesis rate; inhibition of Na+/K+-ATPase; increased cation permeability; inhibition of glucose-6-phosphate-dehydrogenase; inhibition of RNA synthesis; loss of ATP; and covalent binding of steroids to lens proteins(149). Glucocorticoids make stable covalent adducts with the lysine reidues of lens proteins in a non-enzymatic way. These adducts are observed in steroid-induced cataracts, but not in other human cataracts or normal human lenses(150). Although the complication was first documented amongst patients receiving steroids for the treatment of rheumatological diseases such as rheumatoid arthritis it has also been studied in renal transplant recipients(151-154). Nishimoto and colleagues found a 77.8% incidence of posterior subcapsular lens opacities with a mean follow-up period of 25 months. Treatment is not indicated unless patients become symptomatic with glare and impaired visual acuity. An excellent outcome can be expected from cataract surgery.

1.1.3.1.2 Raised Intraocular Pressure and Glaucoma

The association between oral corticosteroids and ocular hypertension is also well recognized and studied (148, 155-158). Steroid-induced ocular side-effects may present as ocular hypertension or open-angle glaucoma depending on the duration of treatment and the susceptibility of the individual. A retrospective epidemiological study of elderly patients on the Quebec health-insurance-plan database demonstrated an increased risk of glaucoma in patients receiving oral glucocorticoids(159). This risk was shown to be dose dependent and to increase with the duration of treatment. The risk of ocular hypertension or glaucoma with oral steroids was found to be lower than

that for ophthalmic topical steroids. Increased intraocular pressure is thought to result from glucocorticoid induced morphological and functional changes in the trabecular meshwork(149). Glucocorticoid effects on trabecular meshwork cells lead to an increased accumulation of polymerized glycosaminoglycans(160) possibly due to decreased availability of catabolic enzymes(161) as well as an increased production of fibronectin and collagen type IV(162).

Raised intraocular pressure is well documented as an ocular complication in renal transplant patients(152, 163, 164).

1.1.3.1.3 Central Serous Chorioretinopathy

Friberg and Eller described serous retinal detachment resembling central serous chorioretinopathy (CSR) following solid organ transplantation(165). Their two patients underwent heart transplantation and subsequently developed bullous, serous detachment of the retina associated with retinal pigment epithelium (RPE) mottling and RPE detachments and demonstrated leakage on fluorescein angiography typical of CSR. There were no other apparent causes for the exudation. Indocyanine green angiography findings in similar patients with CSR are dilated choroidal veins, delayed choroidal filling, intrachoroidal hyperfluorescence, and patchy hypofluorescence at or near the sites of dye leakage examined by fluorescein angiography(166). The literature contains other reports of serous chorioretinopathy following organ transplantation(167) including heart(168, 169) and heart-lung transplantation(170). The heart-lung recipient developed leopard-spot geographical areas of RPE clumping which may have been secondary to localized choroidal intravascular coagulation or to acute hypertension.

The pathogenesis of serous chorioretinopathy is uncertain and there are several theories(171). These include focal loss of RPE barrier function at the leak(172), reversal of the RPE pump at the leak(173), diffuse RPE dysfunction in regions beyond the defect(174) and choroidal abnormalities including increased choroidal permeability and extravascular tissue pressure with dysfunction of the overlying RPE(175-177). Friberg and Eller(165) and others(178) who have reported serous retinal detachments following solid organ transplantation propose that alterations in oncotic pressure gradients and extracellular fluid distribution found in their patients, who all had renal failure, play an important role in the persistence of subretinal fluid when a serous detachment develops. Koyama et al. also describe a case of serous retinal detachment

10 months following renal transplantation but information on renal function and the extent of the detachment was not presented(179).

Systemic steroids have long been associated with RPE damage(180) and their association with serous retinal detachment has been demonstrated in a number of studies(181-185). Reduction of steroid dose appears to lead to recovery(185) with relapse being associated with recurrent steroid therapy(181). The causative mechanisms for these effects have not, however, been explained(186). Increased plasma cortisol concentrations may be responsible for vasospasm or altering permeability in the choriocapillaris in one or more focal areas in the posterior fundus so that large proteins gain entrance to the RPE and subretinal space causing leakage of fluid into the subretinal space (182, 185). Moreover inhibition of collagen synthesis by cortisol may produce thinning of capillary walls resulting in increased capillary fragility. It may be that systemic steroids predispose towards the development of serous chorioretinopathy when there is pre-existing damage to the RPE(187). Idiopathic CSR is self-limited and mild in most patients although approximately 5% of patients have severe permanent loss of central vision because of multiple recurrences over a period of time(182). Interestingly, recovery from serous chorioretinopathy occurs in post transplant patients despite the fact that the steroid dose cannot be promptly reduced because of the risk to the transplanted organ(165, 178, 179).

Another association that has been discussed in the literature is psychological stress(182, 183, 188). Individuals with type-A behaviour have increased levels of plasma cortisol and elevated urinary excretion of free cortisol(184). The role of adrenaline in the development of CSR is supported by the fact that serous chorioretinopathy can be induced in monkeys by prolonged infusion of intravenous epinephrine(176, 177). Stress is certainly a prominent feature in the post transplant period.

The prevalence of these detachments following transplantation is small but Scorolli et al suggest that symptomatic disease may underestimate the problem and propose a study to screen asymptomatic patients post transplant with fluorescein angiography(178).

Medical treatments have no proven influence on the disease. Laser photocoagulation has been used to speed resolution although it does not improve the final visual outcome(172). The results of different studies on this subject are controversial and inconclusive so laser is recommended only in selected cases(184).

1.1.3.1.4 Idiopathic Intracranial Hypertension.

This is an uncommon condition that has also been known as pseudotumour cerebri and benign intracranial hypertension. The condition is characterized by elevated intracranial pressure associated with normal high resolution cranial imaging and normal cerebrospinal fluid composition(189-191). The causative mechanism for idiopathic intracranial hypertension is not known but there are many associations including chronic steroid therapy, increasing steroid dose or recent steroid withdrawal(192-195). Symptoms are those of raised intracranial pressure and the major threat to the patient is loss of vision. Treatment involves removing any postulated precipitating causes, weight loss, acetazolamide, repeated lumbar punctures, shunting and/or optic nerve sheath fenestration. Vision and visual fields are used to monitor the course of disease. Schowengerdt and colleagues(196) reported a case of a paediatric heart transplant recipient who presented with headache and diplopia 3 months after surgery. Examination revealed normal acuity, bilateral sixth nerve palsies and mild optic disc oedema; MRI was normal. The visual acuity deteriorated over the following months and lumbar puncture demonstrated an elevated opening pressure of 530 mm water with normal CSF composition. Despite attempts to both increase and decrease the prednisolone dosage, and further aggressive therapy the patient was left with hand motion vision on the right and light perception on the left. Severe visual deficits occur in 4-12% of patients with idiopathic intracranial hypertension(190). The prevalence of idiopathic intracranial hypertension in a population of paediatric renal transplant recipients was 4.4%(197).

1.1.3.2 Cyclosporine A (CsA/Sandimmune/Neoral)

Visual hallucinations and cortical blindness (198) can occur secondary to CsA treatment as occipital white matter appears to be uniquely susceptible to its neurotoxic effects. CsA has also been associated with disc oedema and idiopathic intracranial hypertension in bone marrow(199) and renal(194) transplant patients and with ischaemic retinal microvasculopathy. Ischaemic retinal microvasculopathy is most often reported after bone marrow transplantation when patients are on CsA having also received conditioning therapy such as busulphan or total body irradiation(200) (199). It has also been reported in renal transplant patients(201).

In most patients the signs and symptoms resolve on reduction or cessation of CsA(202). There is, however, one report in the literature of sudden, complete and irreversible blindness 36 hours after administration of CsA in a kidney-pancreas transplant recipient

(203). In this case the onset of blindness coincided with a sudden elevation in serum CsA levels following intravenous administration.

1.1.3.3 Tacrolimus (Tac/FK506/Prograf)

The ocular toxicity of Tac is similar to that of CsA(204) described above although in one study Tac was shown to be cataractogenic in adult rats (205). This was shown to be due to an accumulation of sorbitol in the lens secondary to a pancreas mediated diabetogenic effect of Tac. A cataractogenic effect has not been seen in humans although post transplant diabetes mellitus does occur.

1.1.3.4 Azathioprine (Imuran)

Ocular toxicity is not a documented feature of azathioprine.

1.1.3.5 Mycophenolate Mofetil (MMF/Cellcept)

Myelosuppression may occur and patients are at an increased risk of opportunistic infection, particularly viral infection. Some authors have reported an increased incidence and/or severity of cytomegalovirus infections with a higher morbidity in renal transplant recipients on MMF(206, 207). This effect appears to be dose related and has not been reported in patients on 2g/day MMF or less.

1.1.3.6 Noninfectious Complications Associated with Underlying Disease Processes

1.1.3.7 Diabetes Mellitus

Diabetes mellitus is a common finding both before and after heart, lung and heart-lung transplantation. Following transplantation it is generally associated with corticosteroid therapy. The principle mechanism of steroid-induced diabetes mellitus is increased insulin resistance.

1.1.3.7.1 Ocular Manifestations

Diabetic retinopathy is the most significant ocular consequence of diabetes mellitus. The pathogenesis is unclear but the earliest histological abnormalities are basement membrane thickening, endothelial proliferation, and loss of pericytes(208). A breakdown of the inner blood retinal barrier and increased retinal blood flow is noted with early retinopathy. Increased blood flow and vascular dilatation are thought to be secondary to altered retinal vascular autoregulation, hyperglycemia and tissue hypoxia. Focal intraretinal capillary closure leads to microaneurysms, retinal vascular loops, dilated capillaries, and ischemic maculopathy. Increased retinal vascular permeability causes dot-blot haemorrhages, macular oedema and hard exudates. Widespread

capillary closure with retinal ischemia occurs with pre-proliferative retinopathy. These changes are noted clinically as venous beading, nerve fibre layer infarcts, and dilated pre-existing intraretinal capillaries (intraretinal microvascular abnormalities). Proliferative diabetic retinopathy is thought to result from a disturbance of the normal complex balance of matrix and growth factors involved in homeostasis of the retinal vessels, including vascular endothelial growth factor, fibroblast growth factors, insulin-like growth factors, platelet-derived growth factor, epidermal growth factor and transforming growth factors(208).

Findings are on a spectrum ranging from mild to severe and are classified as shown in Figure 1.4. The severity of retinopathy correlates with longer duration of diabetes mellitus, younger age at diagnosis, high glycosylated haemoglobin levels, high systolic blood pressure, insulin-dependent diabetes mellitus, proteinuria and small body mass. Blinding complications include vitreous haemorrhage, retinal detachment and neovascular glaucoma(208). Treatment of ocular disease is by tight control of glycaemia, hypertension and hyperlipidemia and photocoagulation for neovascularisation and leakage.

Figure 1.4: Classification of Diabetic Retinopathy[208]

Classification of Diabetic Retinopathy
Non-proliferative (background) diabetic retinopathy
- Mild
- Moderate
- Severe (pre-proliferative)
Proliferative diabetic retinopathy
- Low-risk
- High-risk

1.1.3.8 Hypertension

Systemic hypertension is a common finding both before and after heart, lung and heart-lung transplantation. Following heart, lung and heart-lung transplantation it is generally associated with corticosteroid and CsA immunosuppressive therapy. Mechanisms of drug-induced hypertension are not well understood but CsA induced hypertension may

be due to augmented production of endothelin, impairment of nitric oxide synthesis, neuroendocrine activation, hypervolemia and alterations in vascular reactivity(209).

1.1.3.8.1 Ocular manifestations

The fundus picture in hypertension is related directly to the status of the retinal arteries and the rate of rise and degree of systemic blood pressure(210, 211). Pathologically, the arterioles narrow and develop thickening of their walls as a result of intimal hyalinization, medial hypertrophy and endothelial hypertrophy. The age of the patient may complicate interpretation of the clinical changes as sclerotic changes also occur in the normal aging population. The retinal changes of hypertension may also overlap with those seen with other retinal vascular disease such as diabetes. A classification for hypertensive retinopathy based on recent population based data is shown in Figure 1.5. Secondary ocular complications of chronic systemic arterial hypertension include retinal vascular occlusive disease, macroaneurysm formation, and nonarteritic anterior ischemic optic neuropathy.

Accelerated (malignant) hypertension is a rare and potentially fatal condition leading to changes in the retina, choroid and optic nerve. The acute rise in systemic pressure causes fibrinoid necrosis of the arterioles and papilloedema. Clinically there is focal retinal arteriolar constriction followed by flame retinal haemorrhages, cotton-wool spots, optic nerve oedema and rarely serous detachments. Chronically Elshnig's spots and Siegrist's streaks are seen.

Figure 1.5: Classification of Hypertensive Retinopathy[211]

Grade of Retinopathy	Retinal Signs	Systemic Associations
None	No detectable signs	None
Mild	Generalized arteriolar narrowing, focal arteriolar narrowing, arteriovenous nicking, opacity (copper wiring) of arteriolar wall or a combination of these signs	Modest association with risk of clinical stroke, subclinical stroke, coronary heart disease and death
Moderate	Haemorrage (blot, dot or flame shaped), microaneurysm, cotton wool spot, hard exudate or a combination of these signs	Strong association with risk of clinical stroke, subclinical stroke, cognitive decline, and death from cardiovascular causes
Malignant	Signs of moderate retinopathy plus swelling of the optic disc	Strong association with death

1.1.3.8.1.1 Central Retinal Vein Occlusion (CRVO)

CRVO in heart and heart-lung transplant patients has been described in the literature(212, 213) (214). Patients characteristically present with sudden loss of vision and examination reveals dilated, tortuous veins with retinal haemorrhages in all four quadrants. Occlusion is thought to occur secondary to thrombus formation in the central retinal vein within or near the lamina cribrosa and is associated with systemic abnormalities such as arteriosclerosis, systemic hypertension, diabetes mellitus, hyperviscosity, blood dyscrasias, dysproteinemias, hyperlipidemia, chronic lung disease(215) and vasculitis as well as chronic open angle glaucoma. Patients undergoing cardiopulmonary bypass during heart-lung transplantation may have induced systemic hypothermia and may develop systemic hypotension and raised intraocular pressure. In addition cardiopulmonary bypass is associated with activation of the complement system and alterations in platelet function. These factors have also been associated with ischaemic optic neuropathy as a complication of cardiopulmonary bypass surgery(216). Clotting factors are frequently administered during and after transplantation. Hypertension, diabetes mellitus, hyperlipidemia, chronic lung disease and cardiopulmonary bypass are common in this group of patients and it has been postulated that these conditions may all predispose transplant recipients to CRVO (212).

CRVO may be ischaemic with a risk of rubeosis iridis and glaucoma or non-ischaemic(208). Macular oedema is another sight threatening complication. Treatment is by intraocular pressure control, induction of chorioretinal venous anastomoses and panretinal laser photocoagulation for ischaemic eyes with iris neovascularisation.

1.1.3.9 Horner's Syndrome

Horner's Syndrome occurs when the sympathetic innervation to the eye is interrupted(217). Lesions at any point along the sympathetic pathway result in Horners syndrome. Clinically there is mild-to-moderate ptosis owing to denervation of the sympathetically controlled Müller muscle, an abnormally high lower eyelid 'upside-down ptosis' secondary to involvement of the inferior tarsal smooth muscle, miosis and dilation lag. Depending on the level of the lesion, impaired flushing and sweating may be found ipsilaterally. Anhydrosis affects the ipsilateral side of the body with central, first-order neuron lesions. Lesions affecting second-order neurons may cause anhydrosis of the ipsilateral face. With postganglionic lesions occurring after vasomotor and sudomotor fibres have branched off the sympathetic chain, anhidrosis is

either absent or limited to an area on the forehead. Iris heterochromia is a feature of congenital Horner's or of long-standing or early onset Horner's.

1.1.3.9.1 Anatomy

First-order central sympathetic fibres arise from the posterolateral hypothalamus, descend uncrossed through the mid brain and pons, and terminate in the intermediolateral cell column of the spinal cord at the level of C8-T2 (ciliospinal centre of Budge)(217). Second-order pre-ganglionic pupillomotor fibres exit the spinal cord at the level of T1, enter the cervical sympathetic chain where they are in close proximity to the pulmonary apex and the subclavian artery. The fibres ascend through the sympathetic chain and synapse in the superior cervical ganglion at the level of the bifurcation of the common carotid artery (C3-C4). Post-ganglionic pupillomotor fibres exit the superior cervical ganglion and ascend along the internal carotid artery. Shortly after the post-ganglonic fibres leave the superior cervical ganglion vasomotor and the sudomotor fibres branch off, they travel along the external carotid artery to innervate the blood vessels and sweat glands of the face. The pupillomotor fibres ascending along the internal carotid artery enter the cavernous sinus. The fibres then leave the carotid plexus to briefly join the abducens nerve in the cavernous sinus and enter the orbit through the superior orbital fissure along with the ophthalmic branch of the trigeminal nerve (V_1) via the long ciliary nerves. The long ciliary nerves then innervate the iris dilator and Müller muscle.

1.1.3.9.2 Diagnosis

Clinical examination can be supplemented by pharmacologic testing with cocaine 4% and hydroxyamphetamine 1% drops. 4% cocaine drops will not dilate a Horner pupil. A pre-ganglionic Horner pupil will dilate with 1% hydroxyamphetamine and a post-ganglionic Horner pupil will not.

Second-order pre-ganglionic neuron damage is common following thoracic surgery and chest tube or central venous catheter placement.

1.2 AIMS

This study aimed to prospectively monitor a cohort of patients for the development of ocular complications. These patients were receiving high levels of immunosuppression for the prevention of rejection of heart, lung and heart-lung transplants. As described in the introduction many complications can occur in immunosuppressed patients and many are sight threatening. This study aimed to ascertain the burden of ocular morbidity and the need for screening by monitoring both asymptomatic and symptomatic patients.

1.3 METHODS

1.3.1 Patient Selection

Following local ethics committee approval ocular examination was offered to both symptomatic and asymptomatic patients attending a post-transplantation out-patient clinic at Harefield Hospital between the beginning of June 1999 and the end of August 2000. In-patients were also invited for assessment if they were able to tolerate examination. Repeat examinations were encouraged if symptoms occurred or during follow-up visits.

Most patients were managed on regimes involving steroids, Aza and CsA. A minority of patients were managed on Tac or MMF containing regimes. CMV negative recipients were treated prophylactically with high dose oral aciclovir. CMV antigenaemia was routinely measured in CMV negative patients and treated with IV ganciclovir in the presence of symptoms or other evidence of CMV disease.

1.3.2 Data Collected

The following variables were recorded for each patient: age, gender, date and type of transplant, CMV serologic status of donor and recipient prior to transplant, number of rejections (in the previous 3 months) and immunosuppressive therapy.

Ocular assessment consisted of history and examination with visual acuity and dilated slit lamp examination with direct and indirect ophthalmoscopy using 78D and 28D lenses. Visual acuity was measured using a Snellen chart at a 4 metre distance. Patients who were too unwell to come to the examination room were examined in their beds and a near vision acuity was recorded.

1.4 RESULTS

115 heart, lung and heart-lung transplant recipients were examined. 78 heart, lung or heart-lung transplants were performed at this institution between 1st June 1999 and 1st September 2000. Ocular examination was performed 0 to 1300 weeks (25 years) after transplantation (median 43 weeks). The median age of patients was 51 (range of 16-70). There were 78 men and 37 women. Patients were treated with varying immunosuppressive protocols as shown in Figures 1.6-1.8.

1.4.1 Heart Transplant Recipients

70 heart transplant recipients were examined. The patients ranged in age from 16 to 70 years (54 ± 12.6 years; median ± SD) and consisted of 57 men and 13 women. Reasons for transplantation were idiopathic cardiomyopathy in 31 patients(44%), ischaemic heart disease in 28 patients(40%), congenital heart disease in 4 patients(6%), valvular cardiomyopathy in 2 patients (3%), anthrocycline cardiomyopathy in 1 patient, giant cell myocarditis in 1 patient and unknown in 3 patients. 39 patients (57%) were on treatment for hypertension at the time of ocular examination, 13 patients (19%) were on treatment for diabetes mellitus.

41 of the patients were on oral prednisolone, 57 patients were on CsA based regimes and 11 on Tac based regimes, 54 patients were on azathioprine and 2 on MMF. A number of patients were on anti-microbials with 10 being on septrin, 13 on aciclovir and 1 on nystatin.

Ocular assessment was performed within 1 month of transplantation in 14 patients, between 1 and 6 months in 12 patients and more than 6 months after transplantation in 42 patients (in 2 patients the information was missing) (median ± SD: 17 ± 54 months; range: 1 week to 25 years). Best corrected visual acuities ranged between 6/7.5 and hand movements.

7 patients reported visual symptoms before examination. One patient complained of gritty eyes and no ocular abnormality was seen on examination. Another complained of sticky, bleary eyes and was found to have blepharitis with medial ectropion. Three patients complained of blurred vision: in two patients this was due to cataract and in the third it was due to posterior capsular opacification following cataract surgery. Two patients complained of floaters: one was found to have early lens opacities and a flat pigmented choroidal naevus, the other had mild hypertensive change.

The 14 patients seen within 1 month of transplantation had normal ocular examinations although 1 patient had early asymptomatic cortical lens opacities.

The abnormal findings in the group examined between 1 and 6 months post-transplantation were as follows:

1 patient with a homonymous hemianopia which predated his transplantation,

1 superficial stromal scar in a contact lens wearer,

1 hyperopic patient with narrow angles,

1 patient with prominent optic discs and a history of neuroblastoma,

1 patient with a quiet 1 disc diameter right inferior retinal scar with pre-retinal fibrosis and

1 patient with pigment change along a peripheral vessel in the left eye.

The findings in the patients examined more than 6 months after transplantation were:

1 patient with bilateral madarosis,

1 patient with blepharitis and medial ectropion,

1 bilateral pseudophake and 1 unilateral pseudophake,

10 patients with lens opacities of which 2 had posterior subcapsular opacities and 1 had congenital cataracts,

1 patient with asymmetrical disc cupping,

1 patient with prominent discs who had a history of neuroblastoma,

1 patient with a solitary cotton wool spot,

6 patients with hypertensive retinopathy,

2 patients with minimal non-proliferative diabetic retinopathy,

2 patients with flat pigmented choroidal naevi,

1 patient with bilateral macular RPE disturbance and

2 patients with inferior retinal scars one of whom had bilateral, extensive, peripheral granular RPE disturbance.

None of the patients had any evidence of active inflammation.

Demographic information and ocular findings are shown in Figure 1.6.

1.4.1.1 Repeat Examinations

18 patients had repeat ocular examinations with these taking place between 1 week and 13 months post-transplant (median 3 months). 16 patients were examined twice, one patient received 3 ocular examinations within 6 months of transplantation and another received 4 examinations in the first 6 months post transplantation. The only change in ocular appearance occurred in patient 29 who developed a single blot haemorrhage within 1 month of transplantation. 15 of the 18 patients who had repeat ocular examinations received augmented immunosuppression for rejection episodes and 3 of these also required IV ganciclovir for the management of CMV antigenaemia.

Figure 1.6: Demographic Information and Ocular Findings (Heart Recipients)

Pt. No	Age	Sex	Tx Type	CMV Status Donor/ Recipient	HT	DM	POH	Meds	Time Post Tx (wks)	Ocular Symptoms	VA	
2	56	M	OCTx	N/A	Y	N	Nil	CyA, Aza	43	Nil	4/5; 4/5	Inferior pigmented retinal scar (1.5 DD)
3	63	M	OCTx	+/+	N	Y	Nil	P, Tac, aciclovir	40	Nil	4/6; 4/5	Normal
4	55	M	OCTx	-/-	Y	Y	Nil	P, CyA, Aza	43	Nil	4/5; 4/5	Normal
6	55	M	OCTx	-/+	Y	N	Nil	P, CyA, Aza, aciclovir	35	Nil	4/5; 4/5	Normal
7	35	M	OCTx	-/+	N	N	Nil	P, CyA, Aza, septrin	13	Nil	4/5; 4/5	R inf retinal scar (1 DD) with pre-retinal fibrosis
10	51	M	OCTx	-/-	N	N	Nil	P, CyA, Aza	35	Nil	4/5; 4/5	Normal
13	48	F	HCTx	-/-	N	N	Nil	P, CyA, Aza, aciclovir, septrin, nystatin	5	Nil	4/5; 4/5	Normal
15	45	F	OCTx	+/+	Y	N	Nil		23			
16	41	M	OCTx	+/-	N	N	Nil	P, CyA, Aza	35	Nil	4/5; 4/5	Normal
17	48	M	OCTx	/+	N	N	Nil	P, Aza, Tac	22	Nil	4/6; 4/5	Normal
18	47	M	OCTx	-/+	Y	N	Nil	P, CyA, aciclovir	17	Nil	4/6; 4/5	Normal
21	55	M	OCTx	/-	Y	Y	Nil	P, CyA, Aza, aciclovir	13	Nil	4/5; 4/5	Normal
22	18	M	OCTx	+/-	Y	N	Astig-matism	P, Aza, Tac	13	Nil	4/5; 4/5	Normal

Pt. No	Age	Sex	Tx Type	CMV Status Donor/ Recipient	HT	DM	POH	Meds	Time Post Tx (wks)	Ocular Symptoms	VA	
24	46	M	OCTx	+/-	N	N	Nil	P, CyA, Aza, aciclovir	13	Nil	4/5; 4/6	Pigment change along peripheral vessel LE
26	16	M	OCTx	-/-	Y	N	Nil	P, Aza, Tac, aciclovir	9	Nil	4/5; 4/5	Prominent Discs
27	51	F	OCTx	+/-	Y	N	Myopia	P, CyA, Aza, aciclovir	4	Nil	4/5; 4/5	Normal
29	44	M	OCTx	/+	Y	Y	Nil	P, CyA, aciclovir	1	Nil	4/5; 4/5	Normal
35	48	M	OCTx	+/+	N	N	Nil	P, CyA, Aza	1	Nil	N5; N5	Normal
36	53	M	OCTx	-/-	N	N	Nil	P, CyA, Aza	43	Nil	4/5; 4/5	Normal
38	21	M	OCTx	+/-	N	N	Nil	P, CyA, Aza	1	Nil	N5; N5	Normal
39	30	M	OCTx	-/-	N	N	Corneal abrasion post-op	CyA, Aza	9	Nil	N5; N5	Normal
42	49	F	OCTx	+/-	N	N	Nil	P, CyA, Aza	43	Nil	4/5; 4/5	Disc Asymmetry
44	55	M	OCTx	+/+	N	N	Glaucoma suspect	P, CyA, Aza, aciclovir	4	Nil	4/5; 4/5	Normal
46	53	F	OCTx	+/+	N	N	Nil	P, CyA	17	Gritty eyes	4/5; 4/5	Normal
47	59	F	OCTx	+/+	N	N	Nil	P, CyA, Aza	4	Nil	4/5; 4/5	Normal
48	53	F	OCTx	/+	N	N	Glaucoma suspect	P, CyA, Aza	4	Nil	4/6; 4/6	Normal
49	61	F	OCTx	+/+	N	Y	Nil	P, CyA, Aza, aciclovir	5	Nil	N5; N5	Normal
50	56	M	OCTx	-/-	Y	Y	Nil	P, CyA, Aza, aciclovir	5	Nil	4/5; 4/5	Normal
52	50	F	OCTx	-/	N	N	Nil	P, CyA, Aza, aciclovir	2	Nil	4/5; 4/5	Normal
55	23	M	OCTx	-/+	N	N	Nil	P, CyA, Aza, aciclovir	5	Nil	N5; N5	Normal
57	49	M	HCTx	-/-	N	N	Nil	P, CyA, Aza	5	Nil	4/5; 4/5	ELO (CLO)
59	55	M	OCTx	+/+	Y	N	Nil	CyA, Aza	78	Nil	4/5; 4/5	Normal
62	44	M	OCTx	+/	Y	N	Nil	P, Tac	390	Nil	4/5; 4/5	Normal
63	69	M	OCTx	N/A	Y	N	Nil	CyA, Aza	806	Nil	4/6; 4/5	Normal
64	70	M	OCTx	N/A	Y	Y	Nil	CyA, Aza	702	Nil	4/9; 4/5	ELO (NS)
65	52	M	OCTx	/+	N	N	Myopia	CyA, Aza	494	Nil	4/5; 4/5	Normal
67	56	M	OCTx	-/+	Y	Y	Nil	CyA	196	Nil	4/5; 4/5	Mild NPDR, Mild hypertensive change , PSCLO

Pt. No	Age	Sex	Tx Type	CMV Status Donor/ Recipient	HT	DM	POH	Meds	Time Post Tx (wks)	Ocular Symptoms	VA	
68	56	F	OCTx	/-	N	N	L pseudophakia R Cataract	P, CyA, septrin	36	Blurred vision LE	4/5;HM	R PC-IOL; L white cataract
69	51	M	OCTx	/-	Y	N	Nil	CyA, Aza	286	Nil	4/5; 4/5	Normal
70	60	F	OCTx	/+	Y	N	Nil	CyA, Aza	95	Floaters	4/6; 4/5	Flat, pigmented naevus R fundus, ELO
74	44	M	OCTx	-/+	N	N	Nil	CyA	156	Nil	4/5; 4/5	Congenital cataract (ant polar)
75	55	M	OCTx	/-	N	N	Nil	P, CyA	434	Nil	4/5; 4/5	Moderate hypertensive change
77	34	M	HCTx	+/-	N	N	Nil	CyA, Aza	156	Nil	4/5; 4/5	Bilateral macular RPE disturbance
79	41	M	OCTx	/+	N	Y	Nil	P, Tac, Aza, Septrin	104	Nil	4/5; 4/5	Normal
80	60	M	OCTx	N/A	Y	N	Nil	CyA, Aza	78	Nil	4/5; 4/5	Inferior granular RPE disturbance BEs
81	64	M	OCTx	/+	Y	N	Nil	CyA, Aza	364	Nil	4/5; 4/5	ELO, Moderate hypertensive change
85	29	M	OCTx	-/-	Y	N	Nil	P, Aza, Tac, septrin	88	Nil	4/5; 4/5	Normal
86	58	M	OCTx	/-	Y	N	Nil	CyA, Aza	364	Nil	4/5; 4/5	Madarosis BEs
87	56	M	OCTx	-/+	Y	N	Nil	CyA, Aza	118	Nil	4/5; 4/5	Normal
88	59	M	OCTx	+/+	Y	N	Amaurosis fugax	CyA, Aza	170	Nil	4/5; 4/5	ELO, 1/2 DD flat pigmented naevus
89	57	M	OCTx	N/A	Y	N	Nil	CyA, Aza	546	N/A	N/A	Normal
90	64	M	OCTx	N/A	Y	N	Nil	CyA	546	N/A	N/A	R PSCLO, L ELO
92	65	M	OCTx	N/A	Y	Y	Squint surgery	CyA, Aza	357	N/A	N/A	Solitary CWS
93	42	M	OCTx	-/-	Y	N	Myopia	CyA, Aza	130	Nil	4/5 4/5	Normal
94	70	M	OCTx	N/A	N	N	PC-IOLs BEs	CyA	390	Blurred vision L	4/5 4/5	PC-IOLs BEs, L PCO
95	59	M	OCTx	N/A	Y	N	Nil	CyA, Aza	390	Nil	4/5 4/5	Myelinated nerve fibres
97	62	M	OCTx	+/-	N	Y	Nil	CyA, Aza	128	Nil	4/5 4/5	Normal
98	43	F	OCTx	-/-	Y	N	Nil	CyA, Aza	286	Floaters	4/6 4/6	Mild hypertensive change
100	57	M	OCTx	N/A	Y	N	Nil	CyA, Aza	380	Nil	4/5 4/5	ELO
103	23	M	OCTx	+/+	Y	N	Nil	P, Tac, MMF	130	Nil	4/5 4/5	Normal

Pt. No	Age	Sex	Tx Type	CMV Status Donor/ Recipient	HT	DM	POH	Meds	Time Post Tx (wks)	Ocular Symptoms	VA	
106	47	M	OCTx		Y	N	Nil	CyA, Aza	312	Nil	N/A	Mild hypertensive change
108	67	F	OCTx	N/A	Y	N	TIA with diplopia	CyA	572	Sticky, Bleary eyes	4/54/5	Blepharitis with R punctual eversion
109	64	M	OCTx	-/+	Y	N	Cataract	P, CyA, Aza, septrin	130	Blurred Vision	4/36 4/24	LO BEs (PSCLO & NS)
110	70	M	OCTx	N/A	N	N	Nil	P, Aza	1300	Nil	4/5; 4/5	Normal
111	63	M	OCTx	/+	Y	Y	Nil	CyA, Aza	494	Nil	4/5; 4/5	Mild NPDR
113	49	M	OCTx	-/-	N	N	Contact lens wearer, astigmatism	P, CyA, Aza, aciclovir, septrin, nystatin	6	Nil	4/5; 4/5	R superficial stromal corneal scar
114	56	M	OCTx	+/+	Y	N	L homonymous hemianopia	P, CyA, Aza, aciclovir, septrin	12	Nil	4/5; 4/5	Field defect; Normal ocular exam
115	64	M	OCTx	N/A	Y	Y	DR	CyA, Aza	338	Nil	4/12; 4/5	Mild NPDR
116	60	M	OCTx	N/A	Y	N	Nil	CyA	473	Nil	4/5; 4/5	Blepharitis, Mild hypertensive change
117	38	M	OCTx	-/-	N	N	Nil	P, CyA, MMF, aciclovir, septrin, nystatin	N/A	Nil	4/5; 4/5	Normal

1.4.2 Heart-Lung Transplant Recipients

16 heart-lung transplant recipients were examined. The patients ranged in age from 20 to 58 years (33 ± 12.5 years; median ± SD) and consisted of 10 men and 6 women. Indications for transplantation were bronchiectasis in 12 patients (75%) of which 11 had cystic fibrosis, congenital heart disease in 3 (19%) and primary pulmonary hypertension in 1 (6%). 6 patients (38%) were on treatment for hypertension at the time of ocular examination, 4 patients (25%) were on treatment for diabetes mellitus.

13 of the patients were on oral prednisolone, 9 patients were on CsA based regimes and 6 on Tac based regimes, 9 patients were on azathioprine and 2 on MMF. A number of patients were on anti-microbials with 3 being on septrin and 3 on aciclovir.

Ocular assessment was performed within 1 month of transplantation in 5 patients, between 1 and 6 months in 0 patients and more than 6 months after transplantation in 11 patients (median ± SD: 12 ± 61 months; range: 1 week to 14 years). Best corrected visual acuities ranged between 6/7.5 and 6/18.

7 heart-lung transplant recipients reported ocular symptoms before examination. 4 complained of blurred vision. Two of these patients had posterior subcapsular lens opacities, one had mild non-proliferative diabetic retinopathy with no maculopathy and the fourth had asymmetric disc cupping and mild hypertensive change. One patient had complained of floaters. He was found to have a left Horner's syndrome and no retinal pathology. One complained of intermittent red eyes and one of glare at night. Both these patients had normal ocular examinations.

All of the patients seen within 1 month of transplantation had normal ocular examinations, 1 patient was amblyopic in one eye.

The findings in the patients examined more than 6 months after transplantation were:

4 patients with lens opacities 2 of which had posterior subcapsular opacities,

2 patients with mild non-proliferative diabetic retinopathy,

1 patient with hypertensive retinopathy (this patient also had asymmetric disc cupping),

1 patient with a left Horner's syndrome,

1 patient with bilateral pigment clumping at the macula.

None of the patients had any evidence of inflammation.

Demographic information and ocular findings are shown in Figure 1.7.

1.4.2.1 Repeat Examinations

6 patients had a second ocular examination with this taking place between 1 week and 15 months after transplantation (median 3 months). No patient had a change in ocular findings. One patient had *Aspergillus spp* in his sputum, 2 patients received IV ganciclovir for CMV (one had pneumonitis and the other was treated for antigenaemia) and 3 patients received augmented immunosuppression for rejection episodes.

Figure 1.7: Demographic Information and Ocular Findings (Heart-Lung Recipients)

Pt. no	Age	Sex	Tx Type	CMV Status Donor/ Recipnt.	HT	DM	POH	Meds	Time Post Tx (wks)	Ocular Symptoms	VA	Ocular findings
5	55	M	Heart-lung Tx	+/+	Y	N	Nil	P, CyA,	40	Floaters	4/5; 4/5	Horner's
9	26	M	Heart-lung Tx	-/-	N	N	Myopia	P, Aza, Tac,	30	Nil	4/5; 4/5	Normal
12	24	M	Heart-lung Tx	+/-	N	Y	Myopia	CyA, aciclovir	30	Nil	4/5; 4/5	Mild NPDR
28	42	F	Heart-lung Tx	+/+	N	N	Nil	P, CyA, Aza, aciclovir	4	Nil	4/5; 4/5	Normal
30	31	F	Heart-lung Tx	-/-	N	N	Nil	P, CyA, Aza,	4	Nil	4/5; 4/5	Normal
34	41	M	Heart-lung Tx	+/+	N	N	Nil	P, CyA, Aza	1	Nil	N/A	Normal
45	32	M	Heart-lung Tx	+/-	N	N	Nil	P, CyA, Aza, aciclovir	3	Nil	N5; N5	R amblyopia
54	58	F	Heart-lung Tx	N/A	Y	N	LO	P, Aza	728	Blurred vision	4/5; 4/5	LO (PSCLO BEs)
56	20	M	Heart-lung Tx	+/-	N	Y	Nil	P, CyA, Aza	5	Nil	N5; N5	Normal
58	23	M	Heart-lung Tx	+/-	N	N	Nil	P, Tac, MMF	65	Blurred vision	4/5; 4/12	LO (PSCLO LE)
60	22	M	Heart-lung Tx	/-	Y	N	Nil	Tac, septrin	65	Glare at night	4/5; 4/5	Normal
66	24	F	Heart-lung Tx	N/A	N	N	Nil	P, CyA, MMF, septrin	580	Nil	4/5; 4/5	LO (CLO RE)
72	45	F	Heart-lung Tx	/+	Y	Y	Nil	P, Tac, septrin	565	Red eyes	4/12; 4/12	LO (CLO BEs), Bilateral RPE change at macula, Quiescent scar R peripheral retina
76	34	F	Heart-lung Tx	/+	Y	Y	Nil	CyA, Aza	586	Blurred vision	4/12; 4/12	Mild NPDR
99	54	M	Heart-lung Tx	-/+	Y	N	Nil	P, Tac	442	Blurred vision	4/5; 4/5	Asymmetric cupping, Mild hypertensive change
102	34	M	Heart-lung Tx	N/A	N	N	Myopia	P, Aza, Tac	438	N/A	N/A	Normal

1.4.3 Lung Transplant Recipients

29 lung transplant recipients were examined. The patients ranged in age from 28 to 65 years (52 ± 9.6 years; median ± SD) and consisted of 13 men and 16 women. Indications for transplantation were emphysema in 16 patients (55%) of which 5 had alpha-1-antitrypsin deficiency, bronchiectasis in 7 patients (24%) of which 3 had cystic fibrosis, fibrosing alveolitis in 3 patients (10%), congenital heart disease in 2 patients (7%) and pulmonary fibrosis secondary to busulphan in 1 patient (3%). 15 patients (52%) were on treatment for hypertension at the time of ocular examination, 4 patients (14%) were on treatment for diabetes mellitus.

26 of the patients were on oral prednisolone, 16 patients were on CsA based regimes and 13 on Tac based regimes, 20 patients were on azathioprine and 3 on MMF. A number of patients were on anti-microbials with 12 being on septrin, 10 on aciclovir and 2 on azole antifungals.

Ocular assessment was performed within 1 month of transplantation in 4 patients, between 1 and 6 months in 6 patients and more than 6 months after transplantation in 19 patients (median ± SD: 9 ± 39.2 months; range: 1 week to 11 years). Best corrected visual acuities ranged between 6/7.5 and 6/36.

4 lung transplant recipients reported ocular symptoms before examination. One of these patients complained of a red eye and was found to have a sub-conjunctival haemorrhage. 3 complained of blurred vision. One of these patients had no ocular abnormality. He was advised to see his own optician for refraction. One patient had bilateral soft drusen with no evidence of choroidal neovascularization and the third had posterior subcapsular lens opacities.

One patient seen within 1 month of transplantation had a quiescent central right corneal scar secondary to a corneal ulcer treated 12 years previously and another had a pigmented chorioretinal scar in the left superotemporal periphery.

The findings in the group examined between 1 and 6 months post-transplantation were as follows:

1 patient with uniocular amblyopia,

1 patient with bilateral quiet pseudophakia,

1 with Horner's syndrome and

1 patient with corneal, iris, lens and vitreous changes suggestive of old trauma, although there was no corresponding history.

The findings in the patients examined more than 6 months after transplantation were:

5 patients with lens opacities of which 2 had posterior subcapsular opacities,

1 patient with mild hypertensive retinopathy,

1 patient with Horner's syndrome and a left iris naevus,

1 patient with bilateral quiet pseudophakia and myopic fundal changes,

1 patient with soft drusen,

1 patient with a right peripapillary disciform,

1 patient with large areas of RPE pallor with no pigment clumping and no retinal swelling and

1 patient with a right phthisis.

The patient with the pale fundus gave a history of 2 days of bilateral loss of vision 5 months after transplantation and 4 months before this examination. This was associated with collapse, vomiting, headache and seizures. The patient was on a Tac regime with a level of 11.4 ng/ml (therapeutic range). Examination revealed bilateral blindness and nystagmus with no other ocular or systemic abnormalities. CT scan, lumbar puncture and carotid ultrasound were normal. MRI demonstrated multiple areas of high signal abnormalities in both cerebellar hemispheres and in both parieto-occipital regions. These high signal abnormalities were not present 1 week later. The differential diagnosis was cerebral vascular occlusion or tacrolimus related leukoencephalopathy. Vision returned spontaneously after 48 hours and at examination the visual acuity was 6/7.5 bilaterally. The patient remained on a Tac regime at the time of this examination.

The patient with a right phthisis developed a right endogenous endophthalmitis 2 weeks after transplantation and 8 months before this examination. This was found to be due to *Aspergillus spp.* and although aggressive treatment was systemically successful the eye became phthisical. This patient was an insulin treated diabetic on a MMF regime. She remained on itraconazole and MMF at the time of this examination.

Demographic information and ocular findings are shown in Figure 1.8.

1.4.3.1 Repeat Examinations

5 patients had a second ocular examination with this taking place 1 to 22 months after transplantation (median 8.5 months). One patient developed early posterior subcapsular lens opacities between visits but the others had no change in their ocular findings. One patient had received treatment for intrapulmonary candida, one remained on itraconazole for previous *Aspergillus spp* infection and 3 received augmented immunosuppression for rejection.

Figure 1.8: Demographic Information and Ocular Findings (Lung Recipients)

Pt. no	Age	Sex	Tx Type	CMV Status Donor/ Recipnt	HT	DM	POH	Meds	Time Post Tx (wks)	Ocular Symp-toms	VA	Ocular findings
1	51	F	SLTx	+/-	Y	N	Nil	P, Tac	48	N	4/5; 4/5	LO BEs
8	35	F	DLTx	+/+	N	Y	R endophthalmitis	P, CyA, MMF, itraconazole	35	N	: 4/5	R phthisis with shell
11	53	F	DLTx		Y	N	Nil	P, Tac, aciclovir	30	N	4/5; 4/5	LO BEs
14	54	M	SLTx	-/-	Y	N	Nil	P, CyA, Aza, aciclovir, septrin	5	N	4/5; 4/9	Pigmented CR scar LE, superotemporal periphery 1.5DD
20	45	F	DLTx	-/+	Y	Y	Ametropic amblyopia	P, CyA, Aza, aciclovir	17	N	4/24; 4/18	Amblyopia
23	38	F	DLTx	+/+	Y	N	Nil	P, CyA, Aza, aciclovir	13	N	4/5; 4/5	Normal
25	50	F	SLTx	+/-	N	N	Nil	P, Tac, aciclovir	13	N	4/5; 4/5	Horner's
31	42	F	SLTx	+/+	Y	N	Nil	P, CyA, aciclovir	35	N	4/5; 4/5	Normal
32	57	M	SLTx	+/+	N	N	Left herpes simplex keratitis	P, Aza, Tac	48	N	4/9; 4/5	Normal
33	56	M	SLTx	-/-	N	N	R corneal ulcer	P, CyA, Aza	1	N		R corneal scar
37	52	M	SLTx	+/-	N	Y	Floaters post op	P, CyA, Aza	13	Blurred vision	4/5; 4/5	Normal
40	47	M	DLTx	+/+	N	N	Nil	P, CyA, Aza	40	N	4/5; 4/5	Normal
41	35	F	SLTx	-/-	N	N	Nil	P, Aza, Tac, septin	4	N	4/5; 4/5	Normal
43	55	F	SLTx	-/+	N	N	Squint Sx	P, CyA, Aza, aciclovir	4	N	4/5; 4/5	Normal
51	59	F	SLTx	+/+	N	N	Cortical blindness	P, Aza, Tac	39	Red eye	4/5; 4/5	S/conj haem

Pt. no	Age	Sex	Tx Type	CMV Status Donor/ Recipnt	HT	DM	POH	Meds	Time Post Tx (wks)	Ocular Symp-toms	VA	Ocular findings
53	53	F	SLTx	+/+	Y	N	Nil	P, CyA, Aza	69	N	4/12; 4/5	LO BEs: L Horner's; L iris naevus
71	58	M	SLTx	/-	Y	N	Pseudophakia BEs	P, Aza, Tac, septin,	292	N	4/9; 4/9	PC-IOL BEs; Myopic fundi
73	53	F	SLTx	/+	N	N	Nil	P, Aza, Tac, septrin	494	N	4/9; 4/9	Normal
78	36	M	SLTx	/-	Y	N	Nil	P, CyA, Aza	552	Blurred vision	4/6; 4/5	LO
82	57	M	SLTx	/-	N	N	FB RE	CyA, Aza, septrin, aciclovir	156	N	4/6; 4/6	Normal
83	28	F	DLTx	/-	N	Y	Myopia	P, CyA, MMF, septrin, aciclovir	41	N	4/6; 4/6	Normal
84	34	M	DLTx	+/-	Y	N	Nil	P, Aza, Tac, septrin, itraconazole	312	N	4/6; 4/6	LO
91	43	M	DLTx	+/-	N	N	Nil	Tac, septrin, aciclovir, itraconazole	21	N	4/9; 4/6	L cornea, iris, lens & vitreous changes ? old trauma
96	52	M	DLTx	-/-	Y	N	Nil	CyA, Aza, septrin	286	N	4/5; 4/5	Normal
101	65	M	SLTx	+/	Y	N	Nil	P, Aza, Tac, septrin	386	Blurred vision	4/9; 4/5	Soft drusen BEs
104	57	F	DLTx + ASD repair	N/A	Y	N	Nil	P, Tac, septrin	452	N	4/6; 4/6	Normal
105	39	F	DLTx	/+			Nil	P, Tac, MMF	434	N		Mild hypertensive change

Pt. no	Age	Sex	Tx Type	CMV Status Donor/ Recipnt	HT	DM	POH	Meds	Time Post Tx (wks)	Ocular Symp-toms	VA	Ocular findings
107	63	M	SLTx	-/+	Y		Nil	P. CyA. Aza. septrin	78	N	4/5; 4/5	Right peripapillary disciform
112	46	F	SLTx	+/-	Y		Pseudophakia BEs	P. CyA. Aza	9	N		PC-IOL BEs

1.5 SUMMARY

18 patients (16%) were symptomatic at examination with the most common symptom being blurred vision (Figure 1.9). Of the 115 transplant recipients examined 62 (54%) had findings at ocular examination. The most common findings were cataracts (17% of those examined) followed by hypertensive change (8%), chorioretinal scarring (5%) and diabetic retinopathy (3%). The 6 patients with chorioretinal changes all had quiescent scars. 5 had focal scars, one of which was a peripapillary disciform. One of the patients had bilateral, peripheral, granular scarring inferiorly that would be consistent with previous retinitis but no evidence of active inflammation at the time of involvement in this study. Only one patient was seen to have ocular infective complications following surgery and this was *Aspergillus spp.* endophthalmitis. This patient was treated with amphotericin B but ultimately the eye became phthisical. 33% of asymptomatic patients had ocular findings that may be associated with their immunosuppression (Figure 1.9) compared to 43% of symptomatic patients (Figure 1.10). The most common finding in symptomatic patients was cataract with almost a quarter of patients having posterior subcapsular lens opacity that could be attributed to steroid therapy.

Figure 1.9: Ocular Symptoms in Transplant Recipients

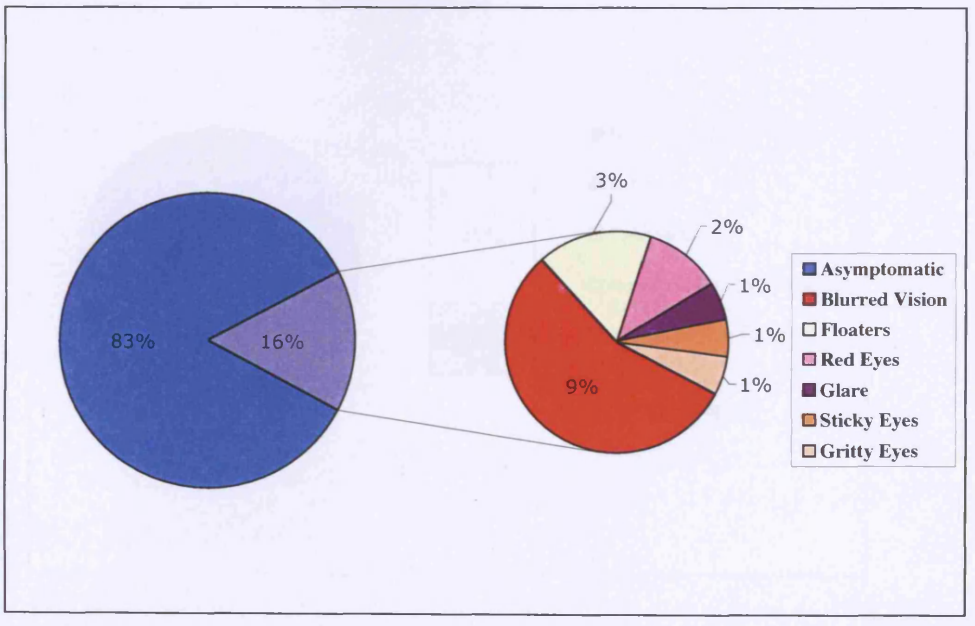


Figure 1.10: Asymptomatic Patients - Ocular Findings that may be Associated with Immunosuppression

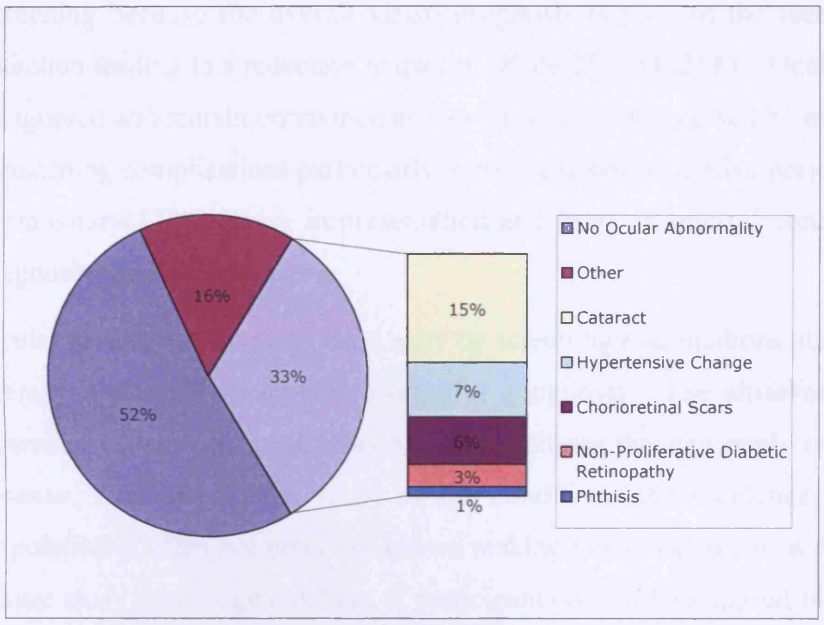
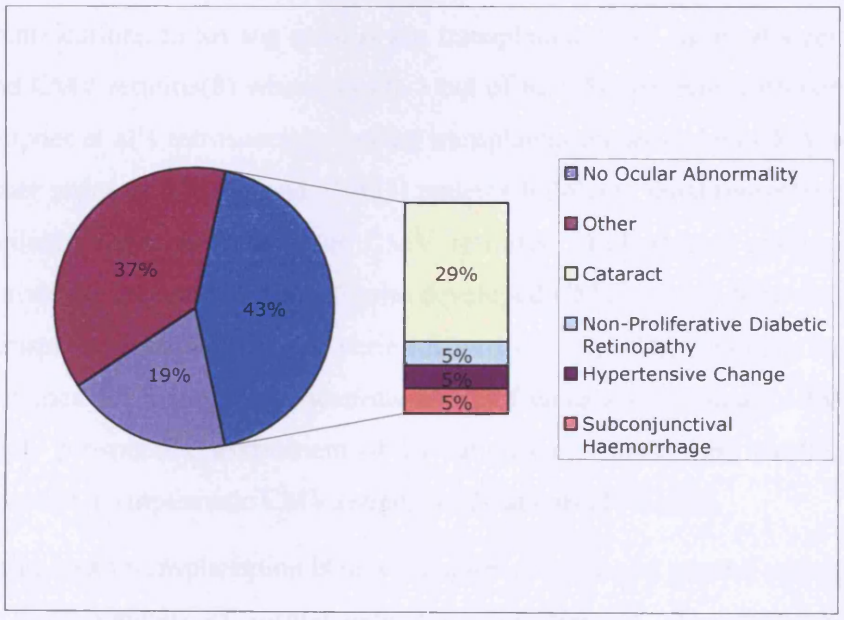


Figure 1.11: Symptomatic Patients - Ocular Findings that may be Associated with Immunosuppression



1.6 DISCUSSION

It has been advocated that solid-organ transplant recipients require regular ophthalmic screening because the overall visual prognosis is poor in the face of opportunistic infection leading to a reduction in quality of life(20, 151, 218). Ocular symptoms may be ignored and remain unreported as they may be overshadowed by concerns about life-threatening complications particularly in the early post operative period and particularly if transitory(178). Delay in presentation and delay in referral contribute to delay in diagnosis and a poor outcome.

Ocular disease can be recognized early by screening examinations allowing for specific therapy and early treatment to improve prognosis. The situation is complicated, however, by the very large array of rare conditions that can rarely cause severe ocular disease. Because of the rarity of the conditions the incidence in the transplant population is often not precisely known making power calculations difficult. A multi-centre study with large numbers of participants would be required to ascertain the risk of ocular disease following transplantation.

A number of small studies have attempted to assess the incidence and morbidity of ocular complications following heart, lung, or heart-lung transplantation with varying results. For example, three out of 19 (16%) patients with symptomatic ocular complications following solid-organ transplantation in Ng et al's retrospective series had CMV retinitis(8) whereas only 3 out of 62 (5%) patients with ocular symptoms in Wapner et al's retrospective cardiac transplantation series had CMV retinitis(219). In other series of 62(152) and 61(153) patients following renal transplantation none of the patients suffered from acute CMV retinitis. Egbert and colleagues at Stanford University reported 11 patients who developed CMV retinitis following heart or kidney transplantation over a 5 year period from 1974(22). Their patients had been routinely screened for ocular complications and half were asymptomatic. In a small Turkish study prospective assessment of 13 patients that underwent cardiac transplantation revealed asymptomatic CMV retinitis in 2 patients (15%)(21).

Solid organ transplantation is now common practice and general screening would result in large numbers of normal patients being screened. Targeted screening has been suggested as a way to minimize the resources required for maximum return. Porter and colleagues suggested screening the subset of patients in whom vigorous and prolonged rejection phenomenon necessitates the persistent use of immunosuppressive drugs in

high doses (151). Cho and colleagues suggest screening the subset of patients with post-transplant lymphoproliferative disorder as 2 of their 3 cases with ocular involvement were asymptomatic (116). Erakgun et al propose CMV screening in the subset of patients with systemic microvascular risk factors such as diabetes, hypertension and smoking(21).

In this study 115 recipients of heart, lung or heart-lung transplants received ocular examinations by a single observer whilst attending a post-transplant clinic or on the ward. Patients were not asked to make extra appointments for ocular examination and the frequency of screening therefore correlated with the frequency of attendance required to manage complications such as rejection or opportunistic infection. Heart, lung and heart-lung recipients were chosen as the study population because they are subject to high doses of immunosuppression for extended periods of time. Ideally patients would have been examined pre-transplant in order to obtain a baseline and then periodically after transplantation for a period of a year. Unfortunately due to the large number of patients awaiting transplant, the scarcity of donors and the speed of transplantation once a donor becomes available this was not done. All patients attending the post-transplant clinic or on the ward were invited for screening examinations. 59 (51%) patients screened were seen for the first time within 12 months of transplantation. 78 transplants were performed at this institution over the 15 months of the study. A number of the patients were being seen regularly in the clinic years after transplant and these patients were included as they were still receiving systemic immunosuppression. 30 (26%) of the patients examined were examined repeatedly over the period of the study providing longitudinal data. Examination would be improved by using LogMar visual acuity assessments and photographic documentation would have made the examination more objective.

18 patients (16%) were symptomatic at examination with the most common symptom being blurred vision. The most common finding in symptomatic patients was cataract with almost a quarter of symptomatic patients having posterior subcapsular lens opacity that could be attributed to steroid therapy. 3 patients in this study had sight-threatening disease that could be attributed to systemic immunosuppression. One patient had fungal endophthalmitis, one had a white cataract and one had bilateral loss of vision which recovered and may have been secondary to drug toxicity. In all three cases the pathology preceded the first examination. This study suggests that the incidence of CMV retinitis after modern immunosuppression is quite low. This is supported by the

fact that a 14 year follow-up of 5721 patients on immunosuppression following haematopoietic stem cell transplantation identified only 10 CMV retinitis cases, one of whom was asymptomatic although asymptomatic individuals were not routinely examined(220). The asymptomatic patient in this case series was being examined for manifestations of graft-versus-host disease. The variations in incidences of CMV retinitis reported post-transplantation are most likely due to centre effects. Because the ocular complications of transplantation are often associated with organ and life threatening events transplant patients are extremely well monitored for many events. Although CMV antigenaemia, does not correlate well with ocular disease(28) improved surgical technique, immunosuppressive regimes and anti-microbial treatment and prophylaxis have changed the natural history of opportunistic infection following transplantation. Prophylaxis is likely to be the reason for the low incidence of herpetic eye disease seen in this study.

The low incidence of common complications such as cataract, diabetic retinopathy and hypertensive retinopathy is surprising. Cataract was the most common ocular complication (17%) and was the most common finding in symptomatic transplant recipients. 4 patients were pseudophakic at examination reflecting access to diagnosis and management of cataract. The low incidence of diabetic and hypertensive retinopathy and the mild signs seen probably reflect the good control and high patient motivation that is required before embarking on transplant surgery.

This study aimed to ascertain the burden of ocular disease following heart, lung and heart-lung transplantation in asymptomatic as well as symptomatic individuals. Sight threatening asymptomatic disease was not seen over the period of study. Thus the study does not support screening of asymptomatic individuals following heart, lung or heart-lung transplantation. Rather, a high index of suspicion is required with prompt and aggressive action when a problem is identified. Patient education must therefore form an important part of any plan to reduce sight-threatening complications. Transplant patients should be encouraged to report promptly if they have ocular symptoms rather like they report exposure to VZV.

CHAPTER 2

QUANTIFICATION OF IMMUNOSUPPRESSION BY FLOW CYTOMETRIC ASSESSMENT OF INTRACELLULAR CYTOKINES – A GENERAL INTRODUCTION

2.1 OVERVIEW OF THE INDUCTION OF THE IMMUNE RESPONSE

Protection against foreign agents involves many different mechanisms some of which are non-specific and some of which are exquisitely specific. Nonspecific defenses are innate and include: mechanical barriers such as the integrity of the epidermis and mucosal membranes, physicochemical barriers such as the acidity of the stomach fluid, antibacterial substances such as lysozyme and defensins present in external secretions, ingestion and elimination of bacteria and particulate matter by granulocytes(221). Specific defenses are induced by the immune response. The immune system is able to discriminate between self and non-self, tolerating self whilst rapidly processing and destroying non-self (foreign) antigens in a primary immune response. In addition, a functioning immune system remembers previous encounters with specific foreign antigens, resulting in a more vigorous and rapid secondary response (immunologic memory).

2.1.1 Cells of the Immune System

2.1.1.1 Lymphocytes

Lymphocytes play a key role in the control and regulation of immune responses, as well as in the recognition of infected or foreign cells which the lymphocytes can recognize as undesirable and thus eliminate. They differentiate from stem cells in the fetal liver, bone marrow and thymus into two main functional classes the B lymphocytes and the T lymphocytes. Lymphocytes are present in bone marrow, lymphoid organs (i.e. thymus, lymph nodes, tonsils, spleen, etc) and peripheral blood. They are categorized by the presence of specific cell surface markers called clusters of differentiation (CD). Each CD marker has a number and the function of a cell can often be predicted by the specific CD markers present.

2.1.1.1.1 B Lymphocytes

The site of B lymphocyte differentiation is unclear and may be the bone marrow or the peri-intestinal lymphoid tissues(222). Human B cells are CD19+ and CD3-. B cells also carry immunoglobulins on their cell membrane which function as antigen receptors. After proper stimulation B cells differentiate into morphologically different antibody producing cells (plasma cells). B cells can also play the role of antigen presenting cells although this function is much more commonly performed by cells of monocytic/macrophage lineage.

2.1.1.1.2 T Lymphocytes

T lymphocytes differentiate in the thymus. They are central to cell mediated immunity and to the regulation of the immune response. Mature human T cells have CD3 on their cell surface (CD3+) and carry T cell receptors on their membranes that are the mechanism for specific antigen recognition. The two main populations are the CD4+ T cells and the CD8+ T cells. CD4+ and CD8+ cells recognize antigens associated with major histocompatibility complex class II and class I molecules, respectively. Other cell markers and cytokine production can be used to further differentiate cells with different effector functions.

CD4+ T cells predominantly have a helper function; proliferating in response to antigen. Upon recognizing an antigen and receiving additional signals from auxiliary cells a small resting T cell rapidly undergoes blastogenic transformation into a large lymphocyte or lymphoblast. This subdivides to produce an expanded population of medium and small lymphocytes with the same antigenic specificity. Activated and differentiated T cells are morphologically indistinguishable from a small resting lymphocyte. A small but important subset, the CD4+ T regulatory cells, has suppressor functions.

CD8+ T cells are capable of killing target cells (cytotoxic T cells) and of downregulating immune responses. T cell mediated cytotoxicity is a complex process involving several possible pathways. Some pathways involve the release of proteins known as perforins which insert themselves in the target cell membranes, forming channels(222). The formation of such channels results in cell death by allowing diffusion of water into the hypertonic intracellular environment causing cellular swelling and eventually loss of integrity. The perforin channels also allow the diffusion of enzymes (serine esterases called granzymes) into the cytoplasm. Once in the cytoplasm, granzymes induce apoptosis, the pathway of which is calcium dependent. They are also involved in a second pro-apoptotic pathway requiring cell-cell contact by way of Fas-Fas ligand receptor contact.

T cells have a longer lifespan than B cells and are important in the development of immunological memory(223).

2.1.1.2 Plasma Cells

These are differentiated B lymphocytes. They are characterized by eccentric nuclei with clumped chromatin and a large cytoplasm with abundant rough endoplasmic

reticulum(222). Plasma cells produce and secrete large amounts of immunoglobulin but do not express membrane immunoglobulins. They divide very poorly if at all and are usually found in the bone marrow and in the perimucosal lymphoid tissues.

2.1.1.3 Natural Killer Cells (NK)

These are large granular lymphocytes(222). They do not carry antigen receptors of any kind but can recognize antibody molecules bound to target cells and destroy those cells using the same general mechanisms involved in T lymphocyte cytotoxicity. They also have a recognition mechanism that allows them to destroy tumour cells and virus-infected cells.

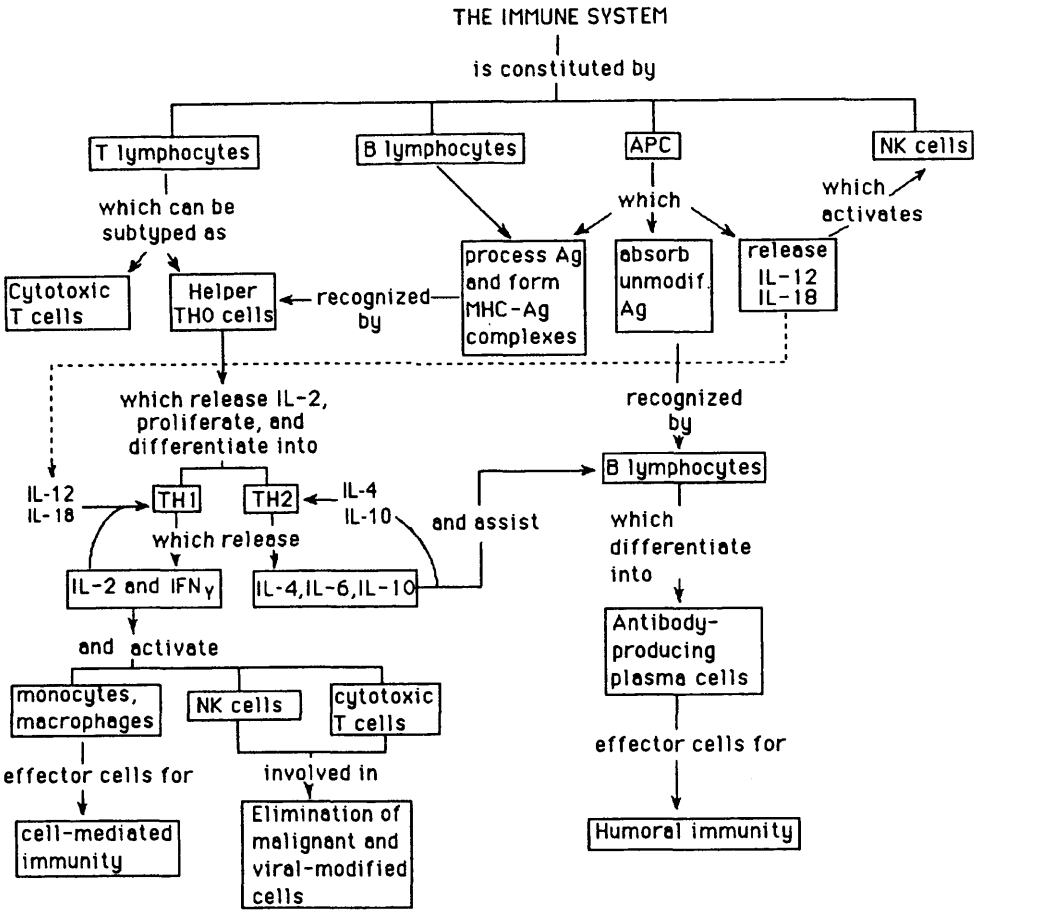
2.1.1.4 Monocytes, Macrophages and Related Cells

Monocytes are leucocytes in transit through the blood and macrophages are monocytes fixed in tissue(222). Blood monocytes may also give rise to dendritic cells in appropriate conditions(224). These monocytes derived cells have phagocytic functions and antigen presenting functions. They process complex antigen and present it on their cell membrane in forms that can be recognized by the appropriate helper T cell thus inducing the immune response. All antigen presenting cells express MHC class II and these histocompatibility antigens are essential for the interaction with helper T cells. Antigen presenting cells also release cytokines such as interleukin 1, -6 and –12 which promote the proliferation of antigen stimulated lymphocytes.

2.1.1.5 Granulocytes (polymorphonuclear leucocytes)

Granulocytes are white blood cells with segmented or lobulated nuclei and granules in their cytoplasm(222). Different subpopulations of granulocytes (neutrophils, eosinophils and basophils) can be distinguished by differential staining of the cytoplasmic granules. Neutophils are the largest subpopulation of white blood cells and have two types of cytoplasmic granules containing bactericidal agents. They are phagocytic and this function is most efficient in the presence of antibody and complement. They are attracted to areas of inflammation by chemotaxis. Chemotactic factors may be released by microbes or may be generated during complement activation as a consequence of an antigen antibody reaction. Eosinophils are found in high concentrations in allergic reactions and during parasitic infections. Basophils are involved in antiparasitic immune mechanisms and play key pathogenic roles in allergic reactions.

Figure 2.1: Overview of the Immune Response[221]



2.1.2 Induction of an Immune response

B cells recognize epitopes expressed by unmodified, native molecules whilst T cells recognize short peptides generated by antigen processing or derived from newly synthesized proteins cleaved in the cytoplasm(225). The immune response is initiated when an antigen or a peptide associated with a MHC molecule is recognized as non-self by immunocompetent cells. The immune system of a normal individual may recognize as many as 10^6 –10^8 different antigenic specificities.

2.1.2.1 Antigen Receptors

In B cells the antigen receptors are membrane inserted immunoglobulins(225). In T lymphocytes the antigen receptors are specific T cell receptors (TcR). Each T cell carries a particular TcR that can recognize peptide complexed with MHC. A number of TcRs can recognise each MHC-peptide complex and any given TcR may be capable of cross-recognising several MHC-peptide complexes. There are two types of TcR. In differentiated T cells the TcR most frequently found is made up of two polypeptide chains designated as alpha and beta with similar molecular weights. A second type of TcR is made up of two different polypeptide chains known as gamma and delta and is predominantly found in submucosal lymphoid tissues. The two chains of the αβ TcR have extracellular segments with variable and constant domains, short cytoplasmic domains and a transmembrane segment. A disulfide bridge joins them just outside the transmembrane segment. The β chains are highly polymorphic and are encoded by a multigene family that includes genes for regions homologous to the V, C, D and J regions of human immunoglobulins. The α chains are encoded by a more limited multigene family with genes for regions homologous to the V, C, and J regions of human immunoglobulins. Similar polymorphisms have been defined for the γδ chains. Together the variable regions of αβ and γδ chains define the highly specific binding sites for peptide epitopes presented in association with MHC II molecules.

2.1.2.2 Antigen Processing and Presentation

Antigen presenting cells (APCs) express MHC II molecules on their membrane where antigen fragments can be bound and presented to lymphocytes(225). Additionally they often express ligands for co-stimulatory molecules and release cytokines that assist the proliferation and/or differentiation of T and B lymphocytes. Antigen processing and presentation is a complex sequence of events that involves endocytosis of antigens on membrane patches and transport to an acidic compartment (lysosome) within the cell that allows antigen degradation into small peptides. As antigens are broken down vesicles coated with newly synthesized MHC-II molecules fuse with the lysosome. Some of the peptides generated during processing have affinity for the binding site located with the MHC-II heterodimer. The resulting MHC-peptide complexes are then transported to the cell surface where they are able to form an immunological synapse with the T cell receptors on T cells.

2.1.2.3 Activation of Helper T cells

The selectivity of the TcR results from selection in the thymus(225). Activation of helper T cells is made specific by the interaction of the TcR and the peptide MHC complex. TcR activation involves a number of signals delivered by accessory cells as well as by the release of soluble factors or cytokines:

1. Signals mediated by CD4-MHC II interactions. The TcR-peptide-MHC II interaction is the only signal in the immune response that is antigen specific. It is of low affinity and other receptor-ligand interactions are required to maintain T cell adhesion to APC and for the delivery of required secondary signals. These receptor-ligand interactions include interactions with nonpolymorphic regions of the MHC molecules: the CD4 molecule on helper T cells interacts with MHC II Molecules while the CD8 molecule on CD8+ cells interacts with MHC-I molecules.

2. Signals mediated by other cell-cell interactions. Several other cell adhesion molecules can mediate lymphocyte-APC interactions for example: CD2 (T cell): CD58 (APC); lymphocyte function antigen (LFA)-1(T cell): intercellular adhesion molecules (ICAM)-1, -2, and –3(APC); CD40L (T cell): CD40 (APC); CD28 (T cell): CD80, CD86 (APC). These interactions are not antigen specific and serve to bring the membranes of the two cells into close proximity. This allows additional activating signals to the T cell and allows for high local concentrations and maximal effects of interleukins and cytokines released by antigen presenting cells and T lymphocytes.

3. Signals mediated by cytokines such as interleukin 1 and interleukin 12.

The TcR heterodimer itself has no recognizable kinase activity. Occupancy and cross-linking of the TcR signals the cell through a closely associated complex of molecules known as CD3 that has signal transducing properties. Co-stimulatory signals are delivered by CD4 as a consequence of the interaction with MHC-II and by CD45, a tyrosine phosphatase activated as a consequence of TcR occupancy. Activation of CD45 initiates the sequential activation of several protein kinases closely associated with CD3 and CD4. The activation of the kinase cascade leads to phopholipase C and other protein kinase activation and results in the mobilization of calcium dependent second messenger systems such as inositol triphosphate. These promote an increase in intracellular free calcium released from intracellular organelles and taken up through the

cell membrane and thus activate a serine threonine phosphatase known as calcineurin. Diacylglycerol (DAG), another product released by phopholipase C, activates another serine/threonine kinase known as protein kinase C (PKC). Multiple other enzymes and adapter molecules are activated in the ensuing cascade.

The activation of second messenger systems results in the activation and translocation of transcription factors such as the nuclear factor-kappa B (NF-κB) and the nuclear factor of activated T cells (NF-AT). Once translocated to the nucleus these factors induce genes controlling cytokine production and T cell proliferation such as those encoding interleukin-2 (IL-2) and the IL-2 receptor.

Activation of the protein kinase cascade also leads to upregulation and modification of several membrane proteins on the T cell membrane such as CD28 and CD40 ligand (CD40L). These molecules interact, respectively with CD80/86 and CD40 on the APC membrane. The interactions involving this second set of molecules deliver additional signals that determine the continuing proliferation and differentiation of antigen-stimulated T cells.

2.1.2.4 Antigen presentation and Activation of CD8+ T Cells

When an APC is infected by an intracellular organism (virus, bacteria, or parasite) the infecting agent will multiply in the cytosol producing its own proteins(225). Some of the microbial proteins diffuse into the cytoplasm where they become associated with degradative enzymes forming a peptide-enzyme complex known as a proteasome. Within these complexes the protein is partially digested and the resulting peptides bind to specialized transporter proteins which deliver them to the endoplasmic reticulum, the site of MHC-1 synthesis and assembly. In the endoplasmic reticulum the foreign/non-self peptides bind to newly synthesized MHC class I molecules and the resulting MHC-peptide complex is transported to the membrane of the infected cell. There the MHC-I peptide complex is presented to a special population of effector T cells capable of killing target cells bearing specific antigen. These T cells are largely CD8+. Resting, circulating CD8+ T cells carry antigen receptors able to recognize associations of MHC-I and non-self peptides; occupancy of the binding site on the TCR by MHC-I associated peptide provides the antigen-specific signal that drives CD8+ T cells. As with T helper cells the stimulation of CD8+ T cells also requires additional signals including cell-cell contact and cytokines. Examples of other important cell-cell

interactions include: CD2(T cell): CD58; LFA-1 (T cell): ICAM family members; CD28 (T cell): CD80/CD86.

The expansion of antigen-activated CD8+ T cells requires the secretion of IL-2. Rarely, activated CD8+ T cells can secrete sufficient quantities of IL-2 to support their proliferation and differentiation and thus proceed without help from other T cell subpopulations. More frequently activated helper T cells provide the IL-2 necessary for CD8+ T cell differentiation.

CD8+ T cells also differentiate and proliferate when exposed to cells from an individual of the same species but from a different genetic background as a consequence of tissue or organ transplantation. In vitro the degree of allostimulation between lymphocytes of two different individuals can be assessed by the mixed lymphocyte reaction. Two types of recognition have been analyzed:

1. Donor peptides presented by nonpolymorphic MHC molecules of donor cells appear to trigger the initial stages of the mixed lymphocyte reaction.
2. As a consequence of the release of cytokines during the initial activation of the rejection reaction the expression of non-self MHC molecules is upregulated on donor tissues. Donor MHC molecules are shed into the circulation taken up by professional APCs of the recipient and processed and presented as non-self peptides associated with self MHC molecules to the immune system of the recipient.

MHC-II expressing cells must be present for the mixed lymphocyte reaction to take place. This requirement suggests that activation of helper T cells by recognition of MHC-II peptide complexes is essential for the differentiation of CD8+ cells. Graft rejection is usually more intense with increasing MHC disparity between donor and host. This is probably due to the greater structural differences between the non-shared MHC molecules. Thus MHC differences are likely to perpetuate the rejection reaction in that self (host-derived) MHC molecules will present non-self peptides derived from the donors MHC molecules.

2.1.2.5 Stimulation of a B cell Response by a T dependent Antigen

In contrast to T cells, B cells recognize external epitopes of unprocessed antigens which do not have to be associated to MHC molecules(225). Some special types of APC such as the Langerhans cells of the epidermis and the follicular dendritic cells of the germinal centers appear to adsorb complex antigens onto their membranes where they are

expressed and presented for long periods of time. Accessory cells and helper T cells provide additional signals necessary for B cell activation, proliferation and differentiation. A complex of proteins is associated non-covalently with the membrane immunoglobulin including CD19 and CD21. These proteins appear to potentiate the signal delivered through occupancy of the binding site on membrane immunoglobulin. Activation and translocation of transcription factors (eg NF-AT, NF-κB) activates the expression of genes coding for immunoglobulin polypeptide chains. For a B cell to complete its proliferation and differentiation into an antibody producing plasma cell or memory B cell a variety of additional signals are required. In the case of the stimulation of a B cell response with a T dependent antigen the additional signals are delivered by helper T cells in the form of both cytokines and interactions between complementary ligands (co-stimulatory molecules) expressed by T cells and B cells. A naïve B cell is initially stimulated by recognition of an epitope of the allergen through the membrane immunoglobulin. Two other sets of membrane molecules are involved in this initial activation: the CD45 molecule and the CD19/CD21/CD81 complex. CD21 is a receptor for C3d a fragment of complement component 3. It is possible that B cells interacting with bacteria coated with C3 and C3 fragments may receive a co-stimulatory signal through the CD19/CD21/CD81 complex.

In the same microenvironment where B cells are being activated, helper T cells are also activated. This occurs because the macrophage can present membrane-absorbed, unprocessed molecules with appropriate immunogenic epitopes to B lymphocytes as well as present antigen in association with MHC-II to helper T cells. Also the B cell may internalize the immunoglobulin-antigen complex, process the antigen and present MHC-II associated peptides to the helper T cells.

The progression of the immune response requires complex interactions between accessory cells (macrophages or B cells), helper T cells, and B cells. The helper T cell receives a variety of co-stimulatory signals from APCs and the activated helper T cell in turn delivers activating signals to APC and B cells. Some of the signals are mediated by interleukins and cytokines such as IL-2 and IL-4 that stimulate B cell proliferation and differentiation, and interferon gamma (IFNγ), which increases the efficiency of APC, particularly macrophages. Other signals are mediated by cell-cell interactions involving CD40L (on T cells) and CD40 (on B cells). As a consequence of signalling through the CD40 molecule, B cells express CD80 and CD86 which deliver

differentiation signals to T cells through the CD28 family of molecules. Additional activation signals are then delivered.

Cell-cell contact also plays a significant role in promoting B cell activation by delivering co-stimulatory signals to the B cell and/or by allowing direct traffic of unknown factors from helper T cells to B cells. Transient conjugation between T and B cells seems to occur constantly due to the expression of complementary CAMs on their membranes. For example, T cells express CD2 and CD4 and B cells express the respective ligands, CD58 (LFA-3) and MHC II; both T and B cells express ICAM-1 and LFA-1, which reciprocally interact.

Once an immune response has been triggered the total number of antigen-specific T and B cell clones will remain the same but the number of cells in those clones will have increased several-fold. It is estimated that the initial helper T cell population is capable of expanding to more than 1000-fold its starting number by day 10. Following such rapid T cell expansion many of the activated effector cells will undergo apoptosis. When the immune response subsides, however, an expanded population of memory T cells will remain and will be able to assist the onset of subsequent responses to the same antigen with greater efficiency and speed which is characteristic of the memory response. As lymphocytes develop into memory cells following antigenic stimulation they cease to express CD45RA and begin to express CD45RO on their surface(226).

2.1.2.6 Regulatory T cells

Regulatory T cells (T reg cells) are a subset of T lymphocytes important for down-grading and thus achieving balance in the immune response. T reg cells express CD4 and CD25(227). Activated non-regulatory cells also upregulate CD25 and T reg cells are characterized further by their expression of the transcription factor *Foxp3* in mice(228) (FOXP3 in humans). It is thought that these cells are likely to represent a separate T cell lineage(229, 230) although in some situations *Foxp3* expression may be induced from naïve T cells after antigen exposure in the periphery. It is likely that several subpopulations of suppressor T cells exist.

The hallmark of the adaptive immune system is the random generation of antigen receptors in developing lymphocyte clones through a process of somatic cell gene rearrangement mediated by the recombination-activating gene recombinase(228). However the diversity of antigen recognition afforded by the system also poses the threat of autoimmunity because of the generation of self-reactive receptors. The

emergence of MHC restricted T cell recognition exacerbates this threat because to develop T cells must express antigen receptors that interact with self peptide MHC complexes. Autoreactive lymphocyte clones are eliminated during development and this clonal deletion together with anergy of self-reactive lymphocytes is known as recessive tolerance. T reg cells further prevent the development of autoimmunity by actively suppressing immune activation, thereby acting as critical mediators of self-tolerance and immune regulation(231).

T reg suppression is mediated by a number of effector mechanisms(232):

1. Cytokine production: IL-10 and TGF-beta have been linked to suppression mediated by T reg cells in in vivo experimental models(233-235)
2. Cell-cell contact: reverse signaling occurs through crosslinking of B7 (CD80 and CD86) on the cell surface of antigen-presenting cells or activated T cells, mediated by CTLA-4 expressed by T reg cells(236).
3. After activation human T reg cells may directly kill activated CD4 and CD8 T cells in a granzyme A and perforin dependent way(237).
4. T reg cells may suppress immune activation by competitive consumption of T cell growth factors such as IL-2(238-240)

Balance is critical and although T reg activity may be usefully responsible for dampening excessive inflammation they may also hamper the effective control of viruses and bacteria. Likewise T reg depletion may usefully lead to more effective immunosurveillance of tumours but may also lead to autoimmunity. The central role of *Foxp3* is demonstrated by the syndrome of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX). This is a fatal human autoimmune disorder in which there are mutations in the gene encoding FOXP3(241). Affected males present with massive lymphoproliferation, early-onset insulin dependent diabetes mellitus, thyroiditis, eczema, severe enteropathy, food allergies, autoimmune haemolytic anaemia and thrombocytopaenia and severe infections. They die between 3 and 4 weeks of age(242). A similar syndrome scurfy occurs in *Foxp3* defective mice(243).

2.2 CYTOKINES

Cytokines are a diverse group of soluble proteins and peptides that act as humoral regulators at nano- and picomolar concentrations(225). They are secreted by a wide range of cells including those of the immune system. Some secreted cytokines also

have membrane-bound forms. Many cytokines are produced by multiple cell types such as lymphocytes, monocytes/macrophages, mast cells and eosinophils and each individual cytokine can have multiple functions depending upon the cell that produces it, the target cell upon which it acts and the other cytokines present in the microenvironment. There is also redundancy in the system with several different cytokines having the same biological function. They can exert their effect through the bloodstream on distant target cells (endocrine), on target cells adjacent to those that produce them (paracrine) or on the same cell that produces the cytokine (autocrine). Physiologically most cytokines probably exert their most important effects in a paracrine and/or autocrine fashion. They exert their effects on target cells by binding to specific high-affinity receptors which in turn initiate intracellular signal cascades, ultimately determining the overall cellular response. The array of biological responses that can be elicited includes activation, differentiation, proliferation, inhibition and apoptosis of immunocompetent cells.

Classification of cytokines is difficult because of their overlapping origins and biological activities. They can be classified according to major origin e.g. lymphokines and monokines or according to major function e.g. interferons, chemokines, colony stimulating factors, tumour necrosis factors and interleukins. No classification is ideal. Most interferons also interfere with virus replication. IFNγ is produced by activated T cells and is an important immunoregulatory molecule. Chemokines are cytokines involved in chemotaxis. The CSFs are so named because they support the growth and differentiation of various elements of the bone marrow. The TNFs are so called because they cause haemorrhagic necrosis of tumours when injected into animals. Interleukins are the largest group and these communicate between various populations of white blood cells. Interleukins are differentiated with numbers. They are produced by a variety of cell types such as monocytes/macrophages, T cells, B cells and even non-leucocytes.

2.2.1 T Helper Subsets

Stimulation of CD4+ T cells in appropriate ways can lead to differentiation into subtypes with polarized cytokine production profiles and effector functions(225, 244-248). T helper 0 (Th0), Th1 and Th2 cells have been defined but these subsets do not have specific membrane markers. Th1 cells produce cytokines such as TNFβ, IFNγ and IL-2 that assist in the proliferation and differentiation of CD8+ T cells and natural killer

cells (cell-mediated immunity); Th2 cells produce IL-4 and/or IL-5 and assist in B cell proliferation and differentiation. Th2 cytokines also promote mast cell and eosinophil proliferation and function. Th0 cells produce both Th1 and Th2 type cytokines. They are postulated to be a transitory precursor cell type but may also be an autonomous cell type resulting from differentiation in the absence of clearly polarizing signals(249). The Th1/Th2 dichotomy was seen first in mice and rats (244, 245, 250) but has also been widely demonstrated in human cells(246, 248, 251).

The type of subtype produced is dependent on(249):

1. The type of antigen presenting cell with macrophages and dendritic cells favouring a Th1 response and B cells a Th2 response.
2. The strength of the interaction between the TCR and peptide. This is dependent on the affinity of the interaction of the TcR with the MHC II associated peptide, the concentration of MHC-peptide complexes on the cell membrane and signals dependent on cell-cell interactions. Strong interactions favour the development of Th1 cells and weak ones the development of Th2 cells.
3. The cytokines present at the time. The early stages of proliferation of the common precursor Th0 cells are IL-2 dependent. IL-2 is the main cytokine released by activated Th0 cells and has both autocrine and paracrine effects thus promoting Th0 proliferation. As the cells continue to proliferate IL-12 promotes Th1 differentiation while IL-4 seems to be crucial for Th2 differentiation. The cellular source of IL-12 is the APCs and experimental work suggests that specific antigens or bacterial products with adjuvant properties may induce the release of IL-12 by those cells thus tilting the immune response towards Th1 differentiation. Cross-regulation of Th1 and Th2 cells through their specific cytokines is also important in determining the nature of the immune response(252). Th1 cytokines including IFNγ are potent inducers of the differentiation of Th1 cells and antagonists of Th2 development and function. On the other hand Th2 cytokines such as IL-10 promote Th2 differentiation whilst inhibiting the production of Th1 cytokines and subsequently down-regulating Th1 driven responses. The Th1/Th2 dichotomy has been shown to be a simplification of the situation and there are very complex networks of cytokine regulatory patterns which are produced in response to, and specifically tailored to, different offending agents.

2.2.2 Interleukin-2 (IL-2) and the Interleukin-2 Receptor (IL-2R)

Two critical early events in T cell activation are the appearance of mRNAs for IL-2 and for the IL-2 receptor in the cytoplasm(225). The upregulation of the IL-2 gene, an essential step for T cell proliferation at the onset of the immune response, requires occupancy of the promoter region by NF-AT/AP-1/NF-κB complexes. The expression of the IL-2 receptor is primarily controlled by NF-κB. The release of IL-2 into the cellular environment of activated T cells expressing IL-2 receptors has significant biological consequences.

IL-2 stimulates lymphocyte proliferation in both autocrine and paracrine loops. Autocrine stimulation involves release of IL-2 from an activated T cell and binding and activation of IL-2 receptors expressed by the same T cell. Paracrine stimulation is likely a consequence of IL-2 overproduction after persistent stimulation of CD4+ T cells. The released IL-2 exceeds the binding capacity of the IL-2 receptors expressed by the producing cell and can stimulate other nearby cells expressing those receptors. The targets of the paracrine effects of IL-2 are CD4+ T cells, CD8+ T cells, B cells and NK cells all of which express IL-2 receptors of varying affinities.

The IL-2R expressed by T and B cells is composed of three different polypeptide chains. The binding of IL-2 to the trimeric high affinity IL-2R induces the association of the IL-2R to specific tyrosine kinases. The end result is the phosphorylation of transcription factors of the STAT family which dimerize after phosphorylation and then translocate to the nucleus. In the nucleus, activated STAT factors upregulate the expression of genes coding for cytokine receptors and induce the appearance of cyclins. As a consequence the cell enters into S phase of the cell cycle. Co-stimulatory signals such as CD86-CD28 interactions are required to sustain this response. IL-2 synthesis and IL-2R expression by CD4+ cells are sustained for about 48 hours to allow clonal expansion of antigen specific CD4+ cells and subsequently downregulated. If the CD86-CD28 interaction is inhibited the T cells become anergic. NK cells constitutively express IL-2 receptors and will secrete tumour necrosis factor alpha (TNFα), IFNγ and GM-CSF in response to IL-2 which in turn activate monocytes.

2.2.3 Pro-inflammatory Cytokines

IL-1, TNFα, IFNγ, IL-6, IL-8 and migration inhibition factor (MIF) are the main soluble factors that influence inflammatory reactions(225). IL-1 and TNFα have

membrane-associated and secreted forms and are involved in the systemic metabolic abnormalities and circulatory collapse characteristic of shock associated with severe infections. They induce acute phase proteins such as alpha1-antitrypsin, fibrinogen, and C reactive protein. They cause the upregulation of cell adhesion molecules, particularly P-selectin and E-selectin in vascular endothelial cells. This upregulation promotes the adherence of inflammatory cells which eventually migrate to the extravascular space where they form tissue inflammatory infiltrates. IL-1 interacts with nuclei in the anterior hypothalamus causing fever (secondarily to stimulation of prostaglandin synthesis) and sleep and increases the production of ACTH. IL-1 exists in two molecular forms, IL-1α and IL-1β encoded by two separate genes and displaying only 20% homology to one another. In spite of this structural difference both forms of IL-1 bind to the same receptor and share identical biological properties. IL-1α tends to remain associated to cell membranes whilst IL-1β synthesized as an inactive precursor is released from the cell after being processed post-translationally by interleukin-converting enzyme. Macrophage derived IL-1 is an important stimulus for the synthesis and secretion of IL-2 by activated T cells.

IL-4 induces IgE synthesis and is enhanced by IL-5. IL-5 drives eosinophilic differentiation and is dependent on IL-4. IL-6, synthesized primarily by monocytes, macrophages, and other APCs has pro-inflammatory and haematopoietic activities. It also induces the synthesis of acute phase response proteins by the liver.

2.2.4 Cytokines with Predominant Immunoregulatory Functions

IFNγ produced by activated CD4+ cells and NK cells has a wide range of effects as nearly all cells express receptors for IFNγ(225). It induces class I MHC antigen expression on all somatic cells and induces class II MHC on APCs thus initiating and perpetuating the immune response. In concert with IL-1β it induces an increase in the expression of ICAM-1 on the cytoplasmic membrane of endothelial cells, enhancing T cell adherence to the vascular endothelium, an essential first step for T lymphocyte migration from the vascular bed. Large numbers of T cells will therefore exit the vascular bed in areas near the tissues where activated T cells are releasing interleukins and will form perivenular infiltrates characteristic of delayed hypersensitivity reactions.

IFNγ stimulates CD8+ and NK cells as well as activating monocytes. After exposure to IFNγ, monocytes undergo a series of changes typical of their differentiation into phagocytic effector cells:

1. The cell membrane becomes ruffled and the number of cytoplasmic microvilli increases by a factor of 10. This change reflects a considerable increase in phagocytic capacities.
2. The expression of MHC class II antigens and Fcγ receptors increases. The increase in the expression of MHC-II enhances the efficiency of monocytes as APCs and the increased expression of Fc receptors further enhances their efficiency as phagocytic cells.
3. The production of cytokines such as TNFα and IL-12 and of several antimicrobial proteins and compounds (including defensins, cathepsins, collagenases, superoxide radicals and inducible nitric oxide synthase) is upregulated. Engulfed organisms are therefore rapidly killed and ingested proteins are efficiently digested in the phagolysosmes. Excessive and protracted production of IFNγ will have adverse effects. Hyperstimulated monocytes may become exceedingly cytotoxic and may mediate tissue damage in inflammatory reactions and autoimmune diseases.

IL-12 is produced by antigen presenting cells after endocytosis of particulate materials or microbes and plays as crucial role in linking innate and adaptive immunity. It induces the production of IFNγ by CD4+ T cells and NK cells at the early stages of the immune response before the differentiation of effector T cells.

IL-18 acts as an IFNγ inducing factor. It is produced and released by APCs and its main targets are Th0/Th1 CD4+ T cells and NK cells.

2.2.5 Clinical Correlations

Th1 type profiles have been found to be typically induced by infection with intracellular bacteria, protozoa and viruses, whereas Th2 type profiles predominate during helminthic infestations and in response to common environmental allergens(247). Strong and persistent Th1 responses have been shown to be involved in organ-specific autoimmunity such as uveitis(253, 254), contact dermatitis and some chronic inflammatory disorders of unknown aetiology. In contrast polarized Th2 responses favour are responsible for triggering allergic atopic disorders(255) such as vernal

conjunctivitis(256) and asthma(257) in genetically predisposed hosts(258). As well as being characteristic of different immunopathological reactions polarized Th1 and Th2 responses are also characteristic of different protective roles. It is hypothesized that a predominance of Th2 cytokines will be protective against Th1 mediated disorders such as allograft rejection(259) and uveitis(260) and conversely, induction of Th1 activity will dampen Th2 mediated actions including hypersensitivity reactions and eosinophilia(252).

Cytokine measurement techniques have been used to provide information on cytokine profiles in clinical disease states(255, 261-264) and to correlate with clinical and laboratory indices. For example Beilin et al found that reduced post-operative pain was associated with an attenuated production of proinflammatory cytokines(265), Jambrik Z et al found a correlation between IL-6 and haemodynamic abnormalities in rabbits with acute pancreatitis(266); Reinisch et al describe a correlation between IL-6 and clinical activity in Crohns Disease(267) and Schuerwegh and co-workers(268) found a correlation between the number of IFNγ producing T helper cells and rheumatoid arthritis disease activity.

2.2.6 Measurement of Cytokines

There are a large number of techniques for the measurement of cytokines(269-271). These include ELISA, ELISPOT, in situ hybridization, immunohistochemistry, limiting dilution analysis, single cell PCR and flow cytometry. Each technique has advantages as well as limitations such as cost, technical difficulty, labour intensive collection and/or analysis and the technique used will depend on the question being asked. Flow cytometry is an ideal technique for the assessment of cytokines in patient samples because it allows the rapid, simultaneous investigation of a number of cellular characteristics including the expression of intracellular markers. In contrast to other techniques individual cells within diverse populations can be assessed, large numbers of events can be collected from each sample and large numbers of samples can be processed. The results can also be quantified.

2.2.6.1 Flow Cytometry

Flow cytometry provides high sensitivity and levels of accuracy. It measures certain characteristics of cells or particles as they travel singly in suspension past a sensing point. The flow cytometer consists of a light source, collection optics, electronics and a computer to translate signals to data. The light source is a laser that emits coherent light

at a specified wavelength. Scattered and emitted fluorescent light is collected by two lenses (one set in front of the light source and one set at right angles) and by a series of optics, beam splitters and filters, specific bands of fluorescence can be measured. This can be used to measure physical characteristics such as cell size and any cell component or function that can be detected by a fluorescent compound can be examined. Multicolour analysis allows the investigation of antigens that co-exist on the same cell and the ability to simultaneously measure multiple parameters on a cell by cell basis makes flow cytometry a powerful tool. In addition, sorting can be used to physically separate a cell or particle of interest from a heterogeneous population. Testing can be rapidly performed on thousands of cells allowing even cells that have only low frequencies to be assessed.

These features make flow cytometry ideal for the quantification of cytokine producing cells and simultaneous immunophenotyping and intracellular cytokine staining identifies the subpopulation responsible for cytokine production(248, 271-275).

2.2.6.1.1 Principles of Flow Cytometry

2.2.6.1.1.1 Optics

The typical optical system consists of a laser that emits a single wavelength of blue light (488 nm) that excites a number of different fluorochromes(276). When conjugated to antibodies, fluorochromes become markers of reactions between the antigen on the cell of interest and the antibody. Cells labelled with the fluorochrome are excited by the laser and the emitted light is detected by using photomultiplier tubes (PMTs) designed to capture only the wavelength of interest. The level of fluorescence can then be determined and displayed. Fluorescence signals above that of autofluorescence and non-specific binding represent cells that are positive for the antigen of interest and those that express only autofluorescence are negative. Thus the percentage of a population of cells that possesses a particular antigen can be determined.

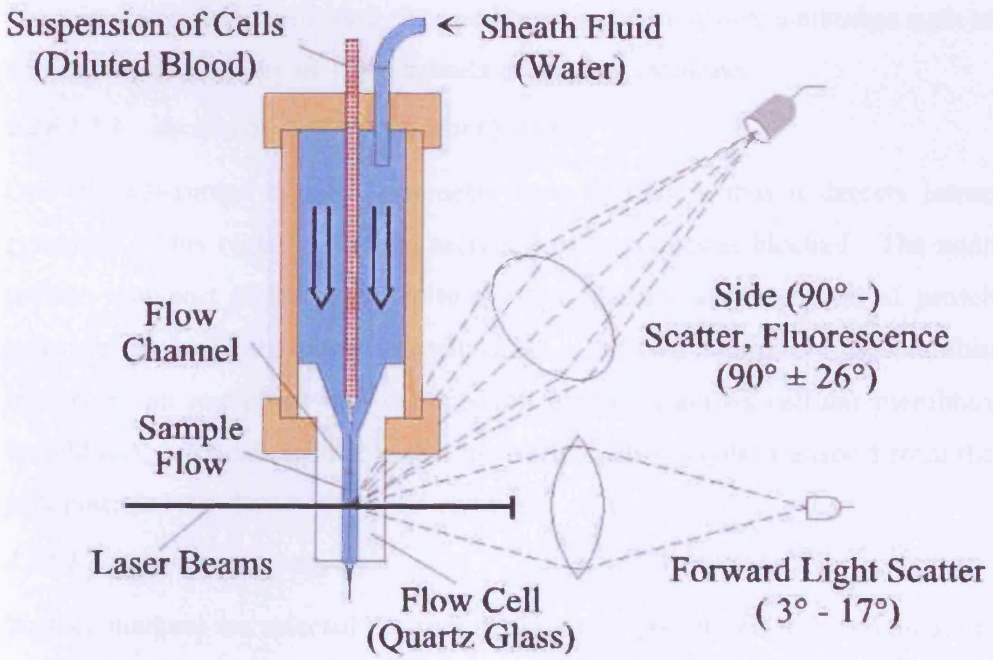
2.2.6.1.1.2 Fluidics

Cells in suspension are measured individually by introducing them into pressurized sheath fluid that travels through a clear cuvette. The cells are focused into a stream in the centre of the cuvette(276) (laminar flow) and this 'hydrodynamic focussing' results in the cells actually passing single-file through the cuvette where the laser light intersects the stream.

2.2.6.1.1.3 Signal Processing

The electronic system measures signals from the PMTs or detectors on a number of parameters on each cell as it passes through the laser light(276). These parameters include fluorescence and light scattering. Forward light scatter is correlated with the size of the cell; side scatter is correlated with the granularity of the cell. The number of fluorochromes detected is limited to the number of fluorochrome-laser light-PMT combinations available. Typically flow cytometers contain three PMTs to detect three fluorochromes simultaneously with one argon laser (488 nm wavelength). Examples of fluorochromes include fluorescein isothiocyanate (FITC), which emits light at 525 nm; phycoerythrin (PE) which emits light at 575 nm; and peridinin chlorophyll protein (PerCP) which emits light at 625nm.

Figure 2.2: Flow Cytometry



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Machine Setup

Instrument setup and compensation are critical for consistent results and must be checked periodically. Thresholds and gates are set to identify the data of interest and to exclude nonviable lymphocytes and cellular debris.

2.2.6.1.2 Activation and Staining

2.2.6.1.2.1 Harvesting Cells

Whole blood or peripheral blood mononuclear cells (PBMCs) may be used for analysis. PBMC are isolated from whole blood using density gradient separation. A whole blood technique may better reflect physiological responses occurring in vivo(277). It has the advantage of maintaining any drug effect during the incubation period as buffering by erythrocytes is maintained and drugs are not washed out.

2.2.6.1.2.2 Activation

The cells are set up in primary culture to induce cytokine production(276). In vitro stimulation of cells is required for detection of cytokines by flow cytometry since cytokine levels are typically too low in freshly prepared cells. This can be done for example with the combination of phorbol esters and a calcium ionophore which induces intracellular signal cascades. The optimal stimulation period for induction of a given cytokine is variable and is determined individually. The short stimulation times required minimise apoptosis and cell proliferation allowing the cellular population that was sampled to be investigated. The addition of costimulatory antibodies such as CD28 enhances the frequency of T cell subsets expressing cytokine.

2.2.6.1.2.3 Accumulation of Intracellular Cytokines

One big advantage of flow cytometry over ELISA is that it detects intracellular cytokines. This requires that the secretion of cytokines is blocked. The addition of protein transport inhibitors leads to the cytoplasmic accumulation of proteins and generates an increased signal intensity(274). The two commonly used inhibitors are monensin, an ionophore that disrupts ion gradients across cellular membranes and brefeldin A, a fungal metabolite that interferes with vesicular transport from the rough endoplasmic reticulum to the Golgi complex.

2.2.6.1.2.4 Immunophenotyping

Surface markers are selected depending upon the type of cell to be studied. T helper cells are identified as CD3+ CD8- cells because of the downregulation of CD4 after phorbol ester stimulation(278).

2.2.6.1.2.5 Intracellular Staining Procedure

The intracellular staining procedure requires the fixation, permeabilization and subsequent staining of the activated cell population with fluorochrome conjugated antibodies. Fixation with paraformaldehyde allows preservation of cell morphology and intracellular antigenicity while also enabling the cells to withstand permeabilization by detergent(279). Membrane permeabilization by saponin allows the fluorchrome-

conjugated cytokine specific monoclonal antibody to penetrate the cell membrane and membranes of the endoplasmic reticulum and Golgi apparatus(280). High affinity anticytokine antibody conjugates with very low levels of background staining are used.

2.2.6.1.3 Controls

The specificity of the anti-cytokine antibody is checked using unlabelled anti-cytokine antibody or isotype control. Unstimulated samples also serve as controls.

2.2.6.1.4 Acquisition and Analysis

The cell population of interest is identified by gating using light scatter and by cellular antigen expression(276). Gated cells are analysed using dual histograms. Quadrants are placed so that the negative populations are on one side of the histogram and the positive ones are on the other. The software can then calculate the proportion of gated cells that are positive for a given antigen.

2.3 LABORATORY TECHNIQUES USED IN THESE STUDIES

2.3.1.1 Cell Stimulation

Peripheral blood was collected in sodium heparin vacutainer tubes and analyzed within 2 hours without refrigeration. A whole blood stimulation method(281, 282) was used on freshly collected cells. 100μl of whole blood was diluted 1:1 with RPMI 1640 (Life Technologies, Paisley, UK) in Trucount tubes(Becton Dickinson, San Jose, CA). Cells were stimulated with phorbol-12-myristate-13-acetate (25ng/ml; PMA; Calbiochem, UK) and ionomycin (1 μg/ml; Sigma; Poole, UK) in the presence of brefeldin A (10μg/ml; BFA; Sigma, UK) which allows cytokines to accumulate within the cell(283). The strength of the TCR-ligand interaction as well as the degree of costimulation will determine the nature of the T cell response. In these studies a strong mitogen stimulation of PMA+ionomycin was used in order to obtain the maximal level of T cell activation for each sample. This was particularly important for reducing variability when comparing repeated measurements over time. In addition CD28 is known to be down-regulated with progression of HIV disease so this was not used in order to avoid variable costimulation in the HIV samples(284). Samples were cultured at 37 C, 5% CO_2 for 16 hours. Unstimulated whole blood samples with BFA added were used as controls.

2.3.1.2 Phenotypic and Cytokine Staining

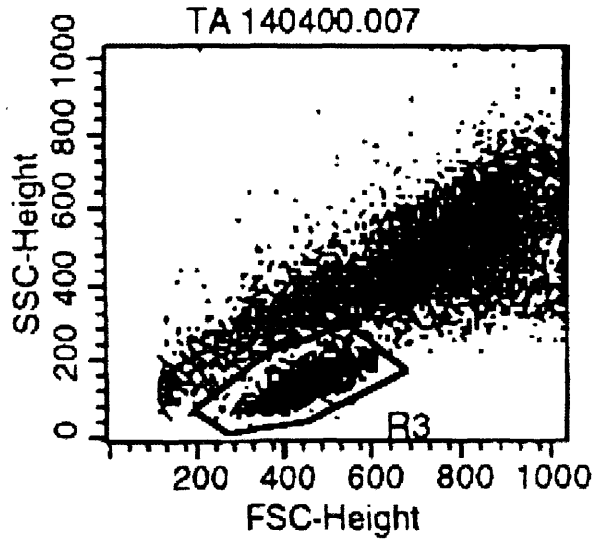
After stimulation blood cells were stained with directly conjugated mabs, anti-CD3 conjugated to FITC or PE and anti-CD8 conjugated to PerCP; (Becton Dickinson, Oxford, UK) 25μl/ml) and incubated for 15 minutes in the dark at room temperature. Red blood cells were then lysed using FACSLyse (Becton Dickinson). The leucocytes were resuspended and fixed and permeabilised using 100μl Cytofix/cytoperm (Pharmingen) for 20 minutes at 4 C. The cells were washed in 1xPermwash buffer (Pharmingen), pelleted by centrifugation, resuspended and stained with previously optimised anti-IL-2-PE (Becton Dickinson), anti-IFNγ-FITC, anti-IL-10-PE (Pharmingen) and anti-IL-4-FITC. In the uveitis study anti-CD69-FITC (Becton Dickinson) was also used. Cells were incubated for a further 30 minutes at 4 C before washing in Permwash, pelleting by centrifugation and final resuspension in FACS Flow (Becton Dickinson). Both Th1 and Th2 cytokine production were assessed. The induction of IL-2, IFNγ, IL-4 and IL-10 were measured in time course experiments to determine the optimal time of PMA stimulation for their detection for use in the study. No one time point was optimal for all the cytokines so the study was focused on IL-2 and IFNγ production which was maximal at 16 hours. At this time point we were unable to demonstrate significant production of either IL-4 or IL-10 in patients or controls and therefore did not look further at these cytokines.

2.3.1.3 Flow Cytometry

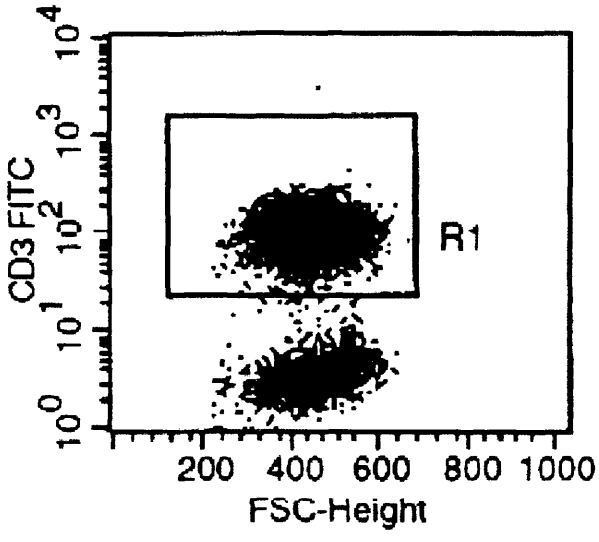
The FACSCalibur flow cytometer (Becton Dickinson) was used for acquisition and data was analysed with CellQuest software (Becton Dickinson). Forward and side scatter profiles were used to identify the lymphocyte region. 10 000 events were collected within this gate for the uveitis patients and a secondary gate set using fluorescent anti-CD3 mab. The number of events collected for the HIV patients was limited by the cell count. As CD4 is known to be down regulated in the presence of phorbol esters, CD8-CD3+ was used to identify CD4 subset(285). Cytokine production by the CD4+ (CD8 negative) and CD8+ T cells at the single cell level was assessed. Results are expressed as the percentage of cells of a particular T cell subset that stained positive for a given cytokine. e.g.: %CD4IL2= CD4+IL2+/[(CD4+IL2+) + (CD4+IL2-)] x 100.

Figure 2.3: Flow Cytometry; Acquisition

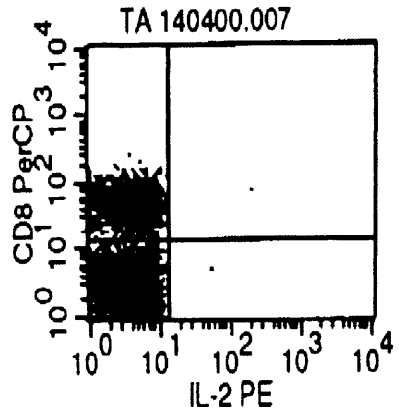
a. **Lymphocytes are gated using size and granularity characteristics**



b. **T lymphocytes are selected using CD3-flourochrome staining**



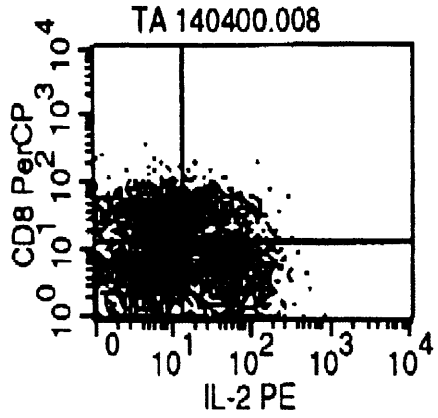
c. Unstimulated T lymphocytes showing no production of IL-2



File: TA 140400.007 Gated Events: 7754

Quad	Events	% Gated
UL	3259	42.03
UR	2	0.03
LL	4492	57.93
LR	1	0.01

d. T cells stimulated with PMA+ionomycin showing production of IL-2



File: TA 140400.008 Gated Events: 6948

Quad	Events	% Gated
UL	2024	29.13
UR	915	13.17
LL	2368	34.08
LR	1641	23.62

CHAPTER 3

ASSESSMENT OF CYTOKINE PRODUCTION

IN

AUTOIMMUNE UVEITIS PATIENTS

ON SYSTEMIC IMMUNOSUPPRESSION

3.1 INTRODUCTION

3.1.1 Definition of Uveitis

Uveitis is a generic term describing inflammation of the vascular, pigmented uvea (iris, ciliary body and choroid) and its adjacent tissues: the retina, retinal vessels, vitreous and sclera. The term gives no clue as to pathogenesis. It has an incidence of only 17/100 000 of the population(286) but is responsible for 10% of those registered legally blind or partially sighted under the age of 65(287). This is likely to be an underestimate as complications of uveitis such as glaucoma, cystoid macular oedema, arterial occlusions and optic atrophy are often recorded on the blind registration form rather than the original diagnosis.

The clinical spectrum is wide and uveitis may occur as an isolated disease or as part of a systemic process. The characteristics of a particular uveitic entity allow it to be put into categories that can provide information on natural history and prognosis for vision. A number of classification systems exist and these are usually used together to characterise a particular condition. The International Uveitis Study Group(288) recommends that patients be classified according to the location of pathology within the eye. Anterior uveitis describes inflammation that is mainly limited to the anterior segment i.e. iritis, anterior cyclitis and iridocyclitis. Intermediate uveitis describes inflammation of the vitreous, pars plana and peripheral retina. Posterior uveitis may be focal, multifocal or diffuse. It is named choroiditis, chorioretinitis, retinochoroiditis, retinitis or neuroretinitis depending on the predominant site of inflammation. Panuveitis describes inflammation throughout the eye. Because the uvea may not always be the initial target of the inflammation an alternative classification system has also been proposed which dispenses with the term uveitis and uses the term intraocular inflammation instead(289). Intraocular inflammation can then be described as being confined to the anterior segment, confined to the posterior segment or present in both parts of the eye.

Clinically the uveitides are subdivided into those with infectious and those with non-infectious causes. Pathologically this is analogous to exogenous or endogenous initiating stimuli. In most, the aetiology is said to be endogenous or autoimmune although it is likely that in some cases where a trigger is thought to be endogenous the particular genetic and environmental background allows micro-organisms to exert an adjuvant like effect. Both infectious and autoimmune uveitides lead to tissue

destruction and scarring and thus to loss of function. Some uveitides are associated with a systemic disease whilst others manifest as idiopathic ocular autoimmune disease.

Posterior uveitis may manifest as a wide range of clinical syndromes. Many of these conditions are discrete clinical entities with well-defined characteristics. Other conditions are less readily diagnosed and pathogenically may represent different aspects of a spectrum of chorioretinal responses that are essentially the same.

3.1.2 Pathology of Posterior Uveitis and Effect on Cytokine Profile

The term posterior uveitis applies to a heterogeneous group of disorders ending in a common final pathway of inflammation, scarring and visual morbidity. Despite much investigation the pathogenesis of posterior uveitis is not well characterised(290) and even within single disease entities it is often difficult to assess the nature of the immune response.

3.1.2.1 Histopathology

Human studies are limited by the paucity of material(290). Sympathetic ophthalmia and Behçets disease are the two examples of panuveitic conditions for which histopathology has been most studied and these will be discussed here as examples of posterior uveitis.

3.1.2.1.1 Sympathetic Ophthalmia

Sympathetic ophthalmia is a classic example of an autoimmune disorder. It is a bilateral inflammatory disease that follows trauma or surgery to one eye(291). Panuveitis occurs and the degree of inflammation and visual prognosis is very variable. The main histopathological features are diffuse granulomatous, non-necrotizing inflammation of the uvea with lymphocytic infiltration of the choroid(292) and Dalen-Fuchs nodules. Dalen-Fuchs nodules are focal collections of epithelioid cells with occasional giant cells and rare plasma cells located between Bruch's membrane and the retinal pigment epithelium(293, 294). Immunocytochemical analysis shows CD4+ T cells dominating early in disease with CD8+ cells becoming more common later(295). B cells have been shown to make up only a small minority of the cellular infiltrate(292, 296, 297) and may correlate with prolonged disease(290).

The traditional hypothesis is that ocular antigens such as on the choroidal melanocyte, which are usually sequestered because of the blood brain barrier, become exposed to the blood stream and to the immune system thereby inciting a uveal autoimmune reaction in the other eye(298). The precise nature of the ocular antigen has still to be determined.

The role of infection in sympathetic ophthalmia continues to be debated. Epidemiological studies have previously shown a higher incidence of sympathetic ophthalmia following accidental (0.19%) rather than surgical (0.07%) trauma and it has been argued that this may be because accidental trauma is more frequently associated with microbial contamination(290). It is postulated that such microbes could be directly infectious, could act indirectly as an adjuvant to upregulate the local immune response or may have structural homology to an endogenous ocular antigen, thus causing disease through molecular mimicry. More recent studies have, however, demonstrated a change in the epidemiology with an increase in the incidence following ocular surgery, in the elderly and in women(299). Sympathetic ophthalmia has been shown to be associated with HLA-DRB1*04 and DQA1*03 in white and Japanese patients (300) and this further suggests that the immune system contributes to disease expression.

3.1.2.1.2 Behçet's Disease

Behçet's Disease (BD) is a chronic, relapsing, multisystem disorder that is characterized by an occlusive vasculitis of unknown aetiology. Typical ocular findings are vitritis, retinitis, optic disc oedema, retinal vasculitis, haemorrhage, oedema and cellular infiltration and these can be associated with blindness in over 70% of the eyes of Behçet's patients(301).

Genetic factors are known to be important with the incidence of BD being markedly different in different ethnic groups. It is relatively common in Japan, for example, where it is responsible for up to 20% of all blindness acquired before middle age. A close association has been found between the HLA-B5101 allele and BD in Japanese and Greek pedigrees(302, 303). However, epidemiological associations have also been found with dental caries, tonsillitis, periodontitis and streptococcal infection and it may be that superantigens can sometimes stimulate the immune response independently of the HLA molecule utilizing the gamma-delta T cell subset(304). Moleular biology techniques have confirmed an association with herpes simplex virus and HSV has been isolated from ocular fluids of patients with BD(305). An animal model exists with HSV inoculated ICR mice showing an induced BD-like syndrome. Histopathological analysis demonstrates a panuveitis with perivasculitis and tissue destruction involving both the anterior and posterior segments although the choroid does not appear to be primarily involved. Inflammatory cells are found in the anterior chamber, corneal endothelium, iris and ciliary body; the retina shows oedema with focal areas of infarction and necrosis and the retinal vessels have thickened, hyalinized walls

infiltrated by lymphocytes(306, 307). The CD4+ T lymphocyte has again been shown to be the major infiltrating cell in blood vessel walls and in the immediate perivascular area(308, 309). A proportion of the retinovascular lymphocytes are activated, expressing IL-2R whereas those found in the anterior segment are IL-2R negative. Studies have reported the presence of B cells, plasma cells(306, 310), eosinophils(311), macrophages(307, 309) and polymorphonuclear cells. IgG, IgA, complement and immune complexes have all been detected(312).

Histopathology has provided important information on pathogenesis but histological and immunohistochemical studies are limited by the fact that they represent single time points in the inflammatory process and these time points are usually at a burnt out, end stage. This may be responsible for apparently conflicting results(290). For example although chorioretinal biopsies of multifocal choroiditis lesions have shown large numbers of B cells in the choroid(313), vitrectomy specimens demonstrated T cell predominance with macrophages making up about one third of the cell population and B cells a rarity(314). A post-mortem case similarly identified 70-80% T cells with around 20% B cells(315) whereas in other reports of multifocal choroiditis the infiltrates were predominantly B cells and plasma cells with some T cells(316). Furthermore in situ immunocytochemical studies of enucleated eyes with a history of chronic inflammation has shown the presence of the cytokines IL-2 and IFNγ which were not present in control eyes with no inflammation(317).

3.1.2.2 Analysis of Peripheral Blood

Increased peripheral T cell activation has been demonstrated in patients with uveitis(318, 319). This is true both for patients with systemic syndromes associated with intraocular inflammation and for patients with intraocular inflammation. In some studies analysis of peripheral blood in patients with BD supports a strong polarization of CD3+ cells to a Th1 type in patients with active disease(254, 319). Frassanito and colleagues also made a correlation between serum IL-12 levels and peripheral Th1 cells and disease progression. Oprempak et al. showed that patients with uveitis had significant frequencies of in vivo activated helper T cells in their peripheral blood as determined by IL-2 analysis(320). Memory and naïve T cells in patients with an underlying systemic disease have been shown to be abnormal although those in patients with idiopathic uveitis did not differ from controls(321).

Abnormal production of various cytokines in the blood appears to be an intrinsic part of many autoimmune disease processes such as rheumatoid arthritis and systemic lupus erythematosis(322). Numerous studies have been done to analyze the cytokine profile of the peripheral blood in uveitis(254, 319, 323-326).

In a study of mRNA from PBMCs, 9 patients with Vogt-Koyanagi-Harada disease (VKH) and 9 controls the PBMCs from VKH patients produced predominantly Th1 cytokines(324). These are postulated to be responsible for the pathological changes. The levels of IFNγ and IL-2 were significantly higher in the stimulated cell culture supernatant of the patients than those of controls and the proportions of IFNγ or IL-2-producing CD4+ cells by flow cytometry were significantly higher in the patients than in controls in both stimulated and unstimulated conditions. However, no significant difference was found in IL-4 producing CD4+ cells.

Likewise, early studies by Ohno et al found that serum levels of IFNγ were significantly higher in BD patients than in normal controls. They also found that the levels of IFNγ varied significantly with stage of disease: higher serum IFNγ levels being seen in patients in the inactive stage of Behçets compared to those in the acute stage(325). More recently, studies using intracellular cytokine staining and flow cytometry have demonstrated increased frequencies of Th1-type CD4+ and CD8+ cells in patients with active BD(254). The frequency of IL-4 producing cells was not increased compared to normals. Sugi-Ikai and colleagues were also able to show that successful treatment with CsA and Tac diminished the frequencies of Th1 type cells whereas unsuccessful treatment did not affect the frequencies of Th1 type cells. They proposed that the levels of Th1 and Th2 type cells in BD could be used as efficient markers for assessment of disease activity and treatment.

In contrast other studies have suggested Th2 type cytokine profiles. For example, one study using ELISA showed that PBMC from patients with BD produced higher levels of IL-4, IL-10, and IL-13 (Th2 cell response), almost normal levels of IL-2, but highly deficient IFNγ and IL-12(327). Another found above normal levels of IL-10 in patients with BD compared to controls associated with low levels of IL-12 using ELISA on previously frozen venous blood samples(328). These results are supported by those that have suggested a marked decrease in CD4+ cells and an increase in CD8+ cells in BD patients with active uveitis(323, 329).

3.1.2.3 Analysis of Aqueous and Vitreous Samples

Analysis of ocular samples aims to give a clearer picture of the in situ disease processes involved in ocular inflammation. Immunocytochemistry of enucleated eyes has clearly demonstrated a central role for the T cell(317) and in particular for the CD4 cell(330, 331) in chorioretinal inflammation. Investigation of aqueous and vitreous samples from patients with uveitis have confirmed that the majority of infiltrating leucocytes are T cells(290, 297, 318, 332-334). T cells in the intraocular fluids of uveitis patients can be shown to be activated and a positive correlation has been made between lymphocyte activation and clinical uveitis activity(318, 332). The proportion of CD4+ T cells and of activated CD4+ T cells in the aqueous humour of patients with anterior uveitis is increased compared with peripheral blood(332, 333, 335, 336). Several studies have demonstrated the presence of cytokines such as IFNγ, IL-2, IL-10, IL-6, IL-12 and TNF-α in the aqueous humour of patients with uveitis although the cellular source of these cytokines was not determined(317, 332, 335-342). Muhaya et al(343) showed that T cell lines derived from vitreous humour cells of patients with uveitis produced significantly higher levels of IL-2, IFNγ and IL-10, but not IL-4, compared with those derived from peripheral blood T cell lines. They also found significantly higher IL-2 production by cells derived from the vitreous of patients with more aggressive intermediate uveitis compared to those patients with the more benign Fuchs heterochromic cyclitis (FHC) ($P = 0.009$). In contrast IL-10 production was significantly higher by the vitreous derived T cells from FHC compared with intermediate uveitis patients. Similar results were found when comparing aqueous humour cytokines in patients with FHC and those with idiopathic anterior uveitis(336). They suggest that high IL-10 production by T cells infiltrating the vitreous of FHC patients could be anti-inflammatory: down-regulating the inflammatory responses and thereby contributing to the more benign clinical course seen in these patients. Lacomba et al(341) also found high levels of IFNγ and IL-2 and low levels of IL-4 in patients with uveitis compared to controls. However, they demonstrate no differences between uveitis and control groups for aqueous IL-10 levels. Hill et al used flow cytometry techniques to demonstrate the cytokines produced by T cells in the aqueous(344). They found increased percentages of interleukin IL-10, but not IFNγ producing T cells in the aqueous humour compared with peripheral blood in patients with acute anterior uveitis, FHC or chronic panuveitis.

These, often conflicting, results make analysis of the disease processes involved difficult. Differences may be related to differences in the activity of the disease or in the populations studied. Although differences in technique and processing are also likely to be responsible for the variations seen it is probable that the cytokine profiles change over the course of the disease process. The diversity and degree of polarization of T cell subsets may reflect the nature of the antigenic and environmental stimuli to which the cells have been exposed, previous antigen exposure, the duration of the inflammation as well as the immunogenetic status of the host(290). It appears that although cytokines direct the course of disease by specifying an initial pathway, the commitment is not rigid and over time there may be a shift from Th1 towards Th2. Despite these problems many investigators have suggested correlations between cytokine production by T cells in uveitis and disease activity or response to treatment. Other cell activation markers such as CD69(345) and ICAM-1(346) have been similarly studied.

3.1.3 Treatment of Posterior Uveitis

Therapy for uveitis is limited by the fact that the immunological mechanisms responsible for ocular inflammation are not clearly defined. Patients are usually managed empirically with non-specific immunosuppressive therapies. An accurate index of diseases activity and a more precise knowledge of the predominant pathophysiological processes would allow more effective evaluation, treatment and follow-up of these diseases(320). Immunosuppression is the mainstay of treatment and aims to dampen the common effector mechanisms of the inflammatory response before irreversible damage occurs. At present treatments are generally non-specific and may therefore have significant systemic side effects associated with them. Medical treatments may be topical, periocular, intraocular or systemic depending on the condition. This discussion focuses on systemic treatment of the posterior uveitides.

3.1.3.1 Systemic Steroid Therapy

Systemic steroids are used in the treatment of bilateral and sight threatening disease(347). The starting dose must be sufficiently high to suppress inflammation and doses of 1mg/kg/day of prednisolone are often used. After the initial high dosage the steroid is tapered slowly titrating clinical response against dose. Reactivation requires a return to high dose. The risk/benefit balance of therapy needs to be discussed with patients before starting treatment and must be monitored throughout treatment. In view

of the possible side effects systemic steroid is not used for inflammation that is not sight threatening. If an infectious agent such as toxoplasma or *mycobacterium spp.* has been identified the relevant anti-microbial cover is required to avoid fulminating infection. Patients are made aware that treatment might be long-term and recurrent. High dose IV methylprednisolone is effective for severe sight threatening uveitis(348) but although the response is rapid, it is short-lived and associated with an increased risk of anaphylaxis, circulatory collapse and death.

3.1.3.2 Second Line Therapy

When doses of more than approximately 10mg/day of oral prednisolone are required to control disease relapse, or if toxicity is limiting adequate dosage for long-term management, then second line steroid sparing agents are needed. Combination therapy allows more effective control of a severe uveitic process as well as allowing the gradual reduction of steroids whilst maintaining a therapeutic remission(349). Drugs used as second line agents in the treatment of uveitis include CsA, azathioprine, methotrexate, mycophenolate mofetil, tacrolimus and more rarely cyclophosphamide and chlorambucil. Unlike steroids these drugs do not have an immediate onset of action and high dose steroids must be maintained until they are effective. Immunosuppressants vary in their mechanisms of action and side effect profiles so treatment must always be tailored to an individual patient and situation.

3.1.3.3 Limitations of Therapy

Often the response to immunosuppressive drugs at high dose is clearly dose dependent but the relationship becomes less clear at lower doses when the disease is coming under control. Maintaining an initially good response whilst reducing the dose may be difficult to achieve. The methods available for the monitoring of immunosuppression help to make therapy safer but are limited by the fact that due to variable pharmacokinetics and pharmacogenetics different patients may have highly idiosyncratic sensitivities to treatment. The relapsing/remitting or chronic nature of many of the posterior uveitis entities necessitates long term or recurrent treatment and the doses required for efficacy may therefore result in cumulative toxicity. Adjustment of dose also depends on clinical response, the most important indicators of which are visual acuity and uveitis activity. Visual acuity may be limited by the complications of uveitis such as cataract, macular scarring or ischaemia in the absence of active inflammation and uveitis activity may be difficult to accurately grade in the chronic phase(345).

3.1.4 Medical Suppression of the Inflammatory Response

Immunosuppressive therapy aims to affect the events of T cell activation pathways and downstream cytokine production and thus modulate the immune response.

3.1.4.1 Glucocorticoids

3.1.4.1.1 Mechanism of Action and Effect on Cytokine Profile

The actions of corticosteroids are divided into mineralocorticoid actions and glucocorticoid actions. It is the glucocorticoid actions that are the mainstay of immunosuppressive and anti-inflammatory therapy and that are used in controlling heightened immunity accompanying transplant rejection, autoimmune diseases and inflammatory disorders(252). The mechanisms of action of glucocorticoids are multi-faceted and incompletely understood. Corticosteroids penetrate the cell membrane and bind with glucocorticoid receptor α forming an intracytoplasmic steroid-receptor complex(350, 351). This complex then rapidly enters the nucleus where it binds to DNA at specific sequences in the promoter region of steroid-responsive genes known as glucocorticoid response elements. The transcription of target genes can then be directly or indirectly regulated. A myriad of metabolic functions are affected involving inflammation, cytokine synthesis and apoptosis(221). The mechanisms used are both direct and indirect and it is likely that more than one mechanism is brought into play depending on the target cell and the specific activation conditions.

Glucocorticoids inhibit leucocyte adhesion to target tissue by down modulating the expression of leucocyte adhesion molecules and leucocyte transmigration to affected tissues(352).

They may have a limited inhibitory effect on early events of TcR/CD3 signal transduction thereby attenuating more distal downstream activation events(252).

They block monocyte-macrophage and T cell derived cytokine production at the transcriptional and post-transcriptional levels(353-355). This involves direct binding to promoter regions of cytokine genes and/or antagonism of nuclear factors such as NF-κB(252, 350). Glucocorticoids probably exert their immunosuppressive effects directly by inhibiting pro-inflammatory Th1 cytokine production and indirectly by sparing or perhaps enhancing the production of Th2 cytokines(252, 356). For example there is an inhibition of IL-12 secretion by monocytes/macrophages and dendritic cells and a

glucocorticoid induced decrease in the ability of T cells to respond to IL-12. IL-12 is a potent differentiation factor for Th1 cells but not for Th2 cells. They also suppress Th1 stimulated IL-1 synthesis by monocyte/macrophages whilst upregulating Th2 supported production of the IL-1R antagonist(252). The promotion of a Th2 cytokine secreting profile by glucocorticoids induces a state of immune tolerance that may persist long after glucocorticoids have been withdrawn.

They have been reported to stimulate the production of transforming growth factor beta (TGFβ1), an immunosuppressive cytokine which inhibits further cytokine production and T cell proliferation(357).

The nitric oxide synthase gene is downregulated leading to reduced release of nitric oxide and less vasodilator effect.

They have been shown to act more distally inhibiting T cell activation through inhibition of signalling events through high affinity cytokine receptors such as the IL2-R(358).

They inhibit T cell activation indirectly by inducing the production of phospholipase A_2 inhibitory proteins, collectively called lipocortins. These proteins control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes by inhibiting the release of their common precursor arachidonic acid from membrane bound stores.

3.1.4.1.2 Side Effect Profile

Little is known about the molecular mechanisms of corticosteroid side effects such as osteoporosis, growth retardation, skin fragility and metabolic effects. These actions of steroids are related to their endocrine effects(350). In general, serious unwanted effects are unlikely if the daily dose is <50 mg hydrocortisone or 10mg of prednisolone or equivalent. A retrospective review of 2111 patients pooled from 71 clinical trials found a relative risk of infections of 1.6 (95% confidence interval, 1.3-1.9) in patients on systemic glucocorticoid treatment(359). The risk was related to the dose with no increased risk of infection being seen in patients given a daily dose of less that 10mg prednisolone a day. Monitoring for the side effects of immunosuppression for patients on oral steroids involves monitoring for end-organ toxicity. In particular, blood glucose, hypertension and bone density are measured. Prophylaxis with

bisphosphonates and/or calcium and vitamin D reduces the risk of osteoporosis in patients on long-term steroids(360).

3.1.4.2 Azathioprine (Imuran)

3.1.4.2.1 Mechanism of Action and Effect on Cytokine Profile

Azathioprine was first synthesized in 1957 as a cytotoxic agent(361). It is an imidazole derivative of 6-mercaptopurine (6-MP) into which it is converted non-enzymatically after oral administration. 6-MP is then enzymatically converted by xanthine oxidase to 6-thiouric acid, by thiopurine S-methyltransferase (TPMT) to 6-methyl-MP, and by hypoxanthine phosphoribosyl transferase (HPRT) to 6-thioguanine (6-TG). It is thought that the 6-TG generated by the HPRT pathway is most likely to mediate the immunosuppressive properties of 6-MP whereas the TPMT pathway is important for azathioprine-mediated side effects(362). Lymphocytes have been shown to enzymatically convert 6-MP to 6-TG. The pharmacologically active 6-mercaptopurine metabolites are known to interfere with DNA and RNA synthesis leading to inhibition of T and B cell proliferation as well as neutrophil production and macrophage activation. Changes in cytokine profile occur secondary to this. The mode of action of azathioprine has been further elucidated more recently(362). Tiede and colleagues have demonstrated that, in addition, the azathioprine metabolite 6-thio-GTP induces T cell apoptosis by specifically inhibiting the activation of Rac1, a GTPase normally activated upon CD28 co-stimulation of T cells. Inhibition of Rac1 target genes including NF-κB and STAT-3 leads to T cell apoptosis. They propose that unchecked proliferation of lymphocytes promotes the development of chronic inflammatory and autoimmune diseases.

3.1.4.2.2 Side Effect Profile

Adverse drug reactions with azathioprine occur in 15-28% of patients(363). The primary toxicity is reversible myelosuppression and epithelial injury, which results in oesophagitis and mild gastrointestinal complaints(351). Hepatotoxicity can occur due to the high rate of RNA synthesis by hepatocytes and acute pancreatitis is reported rarely. Ocular toxicity is not a recognized feature of azathioprine. Monitoring for the side effects of therapy aims to identify end-organ toxicity before it becomes clinically important. Genetic polymorphisms in the TPMT pathway exist which have been shown to result in marked inter-patient variability in the risk of myelosuppression(364). Those with undetectable TPMT activity (1in 300 individuals) have been shown to be at

risk of rapid-onset, prolonged, life-threatening pancytopaenia when treated with conventional doses of azathioprine(363). The use of pretreatment erythrocyte TPMT measurements is therefore advocated, with azathioprine being contraindicated in those with very low or absent TPMT activity. Routine monitoring for azathioprine toxicity involves weekly monitoring of full blood count (FBC) and liver function tests (LFTs) until the maintenance dose is achieved reducing to a minimum of once every 3 months for the duration of therapy.

3.1.4.3 Cyclosporine A (CsA/Sandimmune/Neoral)

3.1.4.3.1 Mechanism of Action and Effect on Cytokine Profile

Cyclosporine A was discovered in 1969 and is an 11 amino acid cyclic polypeptide product of a number of fungi including *Tolypocladium inflatum Gams*(365). It revolutionised solid organ transplantation when it was introduced by providing specific and potent T cell immunosuppression(209, 366, 367). CsA forms CsA-cyclophilin complexes which bind to calcineurin in the cytoplasm(367). Calcineurin normally acts as a serine-threonine phosphatase that dephosphorylates nuclear regulatory proteins such as the NF-AT, facilitating their passage through the nuclear membrane, where they act as transcription factors for the activation of the promoter regions of various cytokines. The CsA-cyclophilin complexes inactivate the enzymatic activity of calcineurin and thus ultimately inhibit the transcription of cytokines namely IL-2, -3, -4 and -5, IFNγ, TNF-α and granulocyte/macrophage colony-stimulating factor. The production of IL-2 and the expression of IL-2R have been demonstrated to be reduced in the peripheral blood of patients undergoing treatment with CsA for disease states(368). In addition CsA enhances the expression of transforming growth factor-β (TGF-β) which inhibits IL-2 stimulated T cell proliferation and the generation of CD8+ T cells. CsA is therefore a potent selective immunoregulator of the cellular arm of the immune response. Evidence also suggests that CsA can inhibit the function of already activated lymphocytes, and is therefore useful in helping to overcome a rejection crisis. The drug appears to be more effective when used with other agents, usually corticosteroids and/or azathioprine and it is often used as a steroid sparing agent, allowing the dose of oral corticosteroids to be reduced.

3.1.4.3.2 Side Effect Profile

Counter balancing the efficacy of CsA is its considerable toxicity. Initially CsA was an oil-based product with significant intra- and inter-individual variation in absorption,

distribution and elimination that complicated its use. Neoral is the modern formulation with CsA incorporated into a micro-emulsion that is readily soluble in fluids, has more predictable pharmacokinetics and can be taken orally. The main problems associated with its use are nephrotoxicity and hypertension. CsA nephrotoxicity may be acute or chronic and appears to be mediated via vasoconstriction of afferent preglomerular arterioles, leading ultimately to necrosis and refractory hypoperfusion. Tremor, hyperlipidemia, gingival hyperplasia and hypertrichosis(365) –including CsA induced trichomegaly(369) can occur. Opportunistic infection, central nervous system toxicity and increased incidence of neoplasm are also significant side effects as discussed below. Ocular toxicity is discussed in Section 1.1.3.2. The therapeutic window is very narrow and therapeutic drug monitoring, for example by radioimmunoassay, is an aid to patient management. Drug levels may be used to monitor the intravenous formulation for toxicity or where drug interactions or altered bioavailability are suspected. They do not have good predictive value. They are used routinely in transplantation medicine to ensure therapeutic levels are reached to prevent rejection. In the treatment of autoimmune disease treatment is usually guided by clinical response. Measurements of blood pressure, creatinine, serum potassium, serum uric acid and LFTs are also required. These can be checked, for example, every 2 weeks for the first 3 months then monthly thereafter. Blood lipids should be measured before treatment and then as appropriate(370).

3.1.4.3.2.1 Opportunistic Infection

Over immunosuppression increases the risk of opportunistic infections. As CsA mainly affects T cell responses with no significant effect on antibody production, the response to bacterial and fungal infection is relatively preserved. CsA has intrinsic antibiotic activity and theoretically should inhibit certain viruses, fungi, protozoa and helminthes but in practical terms these effects are insignificant (209).

3.1.4.3.2.2 Neurotoxicity

Between 10-28% of patients on CsA experience neurotoxic symptoms such as tremor, neuralgia or peripheral neuropathy(371). Severe symptoms affect up to 5% of patients and include psychoses, seizures, leukoencephalopathy, visual hallucinations and cortical blindness (198). Calcineurin inhibition by CsA alters sympathetic outflow which may play a role in the mediation of neurotoxicity. Injury to both the major and minor vasculature may cause hypoperfusion or ischemia and local secondary toxicity in the white matter. Neurotoxicity is associated with severe, diffuse abnormalities of the

white matter on MRI. In most patients the signs and symptoms resolve on reduction or cessation of CsA(202).

3.1.4.3.2.3 Malignancy

CsA immunosuppression increases the risk of malignancies, especially those implicated with a viral aetiology such as lymphoma and Kaposi's sarcoma(209). This mechanism is supported by the observation that in many patients regression is seen after discontinuation or reduction in the dose of CsA.

3.1.4.4 Tacrolimus (Tac/ FK506/Prograf)

3.1.4.4.1 Mechanism of Action and Effect on Cytokine Profile

Tac is a hydrophobic macrocyclic lactone derived from *Streptomyces tsukubaensis(367, 372)*. After binding to intracellular binding proteins (FKBP-12, -13, -25 or -52) the resulting dimer engages the calcineurin/calmodulin/calcium complex, thereby inhibiting calcineurin phosphatase activity(373). This affects cytokine expression and Tac therefore blocks IL-2 production and IL-2R expression by activated T cells in a similar way to CsA although it is not related to it structurally. In vivo and in vitro models of immunosuppression have shown Tac to be up to 100 times more potent than CsA(372).

3.1.4.4.2 Side Effect Profile

Nephrotoxicity (thrombotic microangiopathy), neurotoxicity or gastrointestinal toxicity are the most common adverse effects of Tac and alopecia has also been documented(374). Ocular toxicity is discussed in Section 1.1.3.3. Tac has been shown to be diabetogenic although this may not be clinically significant in the majority of patients(375). Like CsA, Tac can be used as a steroid-sparing agent. Inter-patient variability in Tac pharmacokinetics makes therapeutic drug monitoring an important adjunct to treatment. Although there is poor correlation between Tac levels and drug efficacy, the relative risk of toxicity is increased with higher trough concentrations. Serum creatinine and serum potassium must be measured regularly. Cardiomyopathy has been reported in children on Tac so monitoring of cardiac status with electrocardiograms and ECHO is important(370).

3.1.4.5 Mycophenolate Mofetil (MMF/Cellcept)

3.1.4.5.1 Mechanism of Action and Effect on Cytokine Profile

Mycophenolate mofetil is a prodrug that is hydrolyzed by liver esterases to mycophenolic acid (MPA). MPA is an anti-proliferative agent that is a potent, non-

competitive reversible inhibitor of inosine 5'-monophosphate dehydrogenase(367, 376, 377). This is the rate-limiting enzyme in the de novo pathway for purine synthesis. Resting lymphocytes rely on a salvage pathway involving the enzyme hypoxanthine-guanine-phophoribosyl-transferase for purine biosynthesis whereas activated lymphocytes are dependent on the de novo pathway. MPA suppresses T and B cell proliferation more potently than that of other rapidly dividing cells such as neutrophils and erythrocytes that can use the salvage purine synthesis pathway instead. Changes in the cytokine profile will occur secondarily to the inhibition of proliferation. Because MMF is relatively selective for lymphocytes it has fewer side effects than other immunosuppressive drugs. Its effects are rapidly reversible upon discontinuation(376). An additional immunosuppressive effect of MMF on impairing APC function in dendritic cells has also been demonstrated(378).

3.1.4.5.2 Side Effect Profile

The most common side effects of MMF therapy are gastrointestinal such as nausea, vomiting and diarrhoea. Myelosuppression with neutropaenia may also occur and patients are at an increased risk of opportunistic infection, particularly viral infection. Some authors have reported an increased incidence and/or severity of cytomegalovirus infections with a higher morbidity in renal transplant recipients on MMF(206, 207). Ocular toxicity is discussed in Section 1.1.3.5. Monitoring for toxicity involves FBC weekly during the first month, twice monthly in months 2 and 3 and then monthly throughout the first year(370).

3.2 AIMS

The management of autoimmune disease is often hampered by problems with assessing the level of inflammation. This is particularly true in chronic ocular disease where media opacities, scarring and oedema may limit visual acuity and make clinical assessment difficult. This study aimed to evaluate cytokine measurements as an indicator of the efficacy of immunosuppression and thus as a means of titrating drug dose. If shown to be quantitative and to correlate with dosage, cytometric assessment of intracellular cytokines could improve the accuracy of clinical monitoring.

3.3 METHODS

3.3.1 Patient Selection

The study was open to all patients on or requiring systemic treatment in the uveitis clinic at Moorfields Eye Hospital. 62 patients requiring systemic immunosuppression were recruited and informed consent was obtained following local ethics committee approval. Blood samples were taken for analysis by flow cytometry at standard outpatient clinic visits.

3.3.2 Data Collected

Patient characteristics recorded at each visit included age, sex, uveitis diagnosis and drug history. Ophthalmology examination including visual acuity testing, tonometry, slit-lamp examination and indirect ophthalmoscopy was performed. Clinical activity at each visit was assessed using Snellen visual acuity, the presence or absence of cells and flare in the anterior chamber, keratic precipitates, the presence or absence of cells and haze in the vitreous, the presence or absence cataract and the presence or absence of cystoid macular oedema. Anterior chamber cells, flare, and vitreous haze were each graded 0-4. These findings, together with comparisons with previous examinations were used to classify patients as having active or inactive disease. The International Uveitis Study Group Classification was used for disease classification(288).

Patients were divided into sub-groups for analysis according to their immunosuppressive drug regime, whether or not their disease was considered to be active and associated systemic disease (see Figure 3.1). Comparisons were made between cytokine expression in the patient groups and that in samples from healthy

laboratory workers ("Normals"). Samples were requested from patients on multiple occasions.

Cell stimulation, phenotypic and cytokine staining and flow cytometry were performed as described in Section 2.2.7.

3.3.3 Statistical Analysis

Statistical calculations were performed using SPSS for Windows (version 9.0-12.0 SPSS Inc, IL, USA). Non-parametric Kruskal-Wallis testing was used with Mann-Whitney testing to locate differences. In order to limit the number of comparisons Mann-Whitney testing was only used if a difference was seen with the Kruskal-Wallis test. Results are presented in Figures 3.5-3.21. "N/A" denotes not applied and "Sig." denotes significance. $P<0.05$ was taken to be significant. A Bonferroni correction was used for multiple pairwise comparisons. Boxplots show the median, interquartile range, outliers and extreme cases. When analyzing for the effect of drug regime general linear model multivariate analysis was used with Tukey's honestly significant difference as a correction for the larger number of multiple comparisons. Correlations were assessed by calculating Spearman's rank correlation coefficient.

In those patients providing repeat samples cytokine production at the first and second visit was compared using the Wilcoxon test for 2 related samples.

Figure 3.1: Groups for Analysis

ANALYSIS	GROUP
I	1. Normals
(1st Visit)	2. Uveitis Patients
II	1. Normals
	2. No Immunosuppression (active)
(1st Visit)	3. Active on Systemic Rx
	4. Controlled on Systemic Rx
III	1. Normals
(1st Visit)	2. Ocular Syndrome
	3. Systemic Disease
	1. Normals
	2. No Immunosuppression (active)
IV	3. Steroids only
(1st Visit)	4. Steroids + CyA
	5. Steroids + Aza
	6. Steroids + Cellcept
	7. Others

3.4 RESULTS

61 patients (35 female, 26 male) were analyzed. 1 patient was not analyzed because all his clinical and laboratory data was missing. Missing values for other individuals were included in the analysis. The median age was 49 years (range 16-78 years) (see Figure 3.2.) Six patients had anterior uveitis with 3 of these having chronic anterior uveitis. 8 had idiopathic intermediate uveitis. 15 had posterior uveitis with 4 of these patients having idiopathic retinal vasculitis, 4 birdshot chorioretinitis, 2 toxoplasmosis, 1 bilateral acute retinal necrosis, 1 unspecified retinitis and 2 patients developed CMV retinitis whilst on systemic immunosuppression for treatment of systemic conditions (systemic lupus erythematosus and renal transplantation). 28 patients had panuveitis with 10 of these having a diagnosis of systemic Behcet's, 4 sarcoidosis and 2 Vogt-Koyanagi-Harada's syndrome. HIV status was not checked but none of the patients in this study had suggestive clinical features. Figures 3.3 and 3.4 are graphic representations of patient diagnoses. The visual acuity in each eye ranged from 6/5 to NPL with a median of 6/12. Results were compared to those obtained from 15 healthy laboratory workers ("Normals"; ages 22-47, median 31, 7 males, 8 females) and between patient sub-groups. In each analysis Group 1 is the Normal group unless otherwise stated. Results are limited to the type 1 cytokines IL-2 and IFNγ as neither IL-4 or IL-10 expression were detected in patients and Normals.

Figure 3.2: Age Distribution of Patients

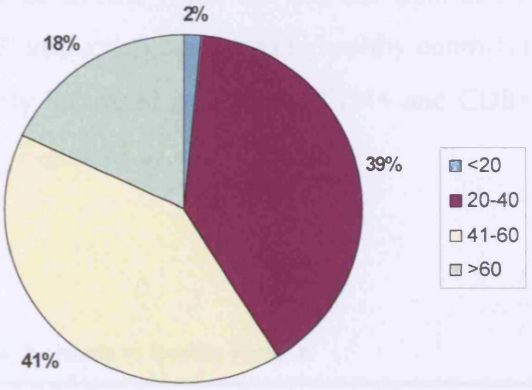


Figure 3.3: Distribution of Patients by Uveitis Classification

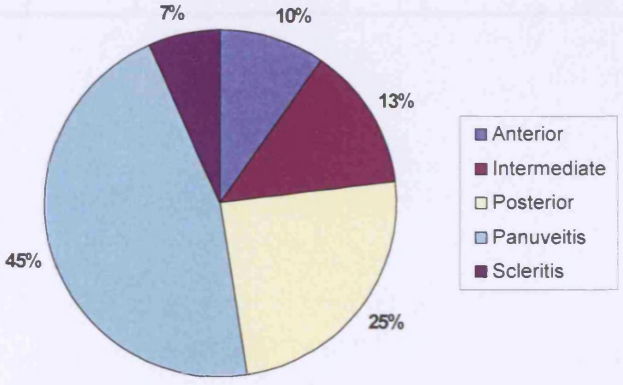
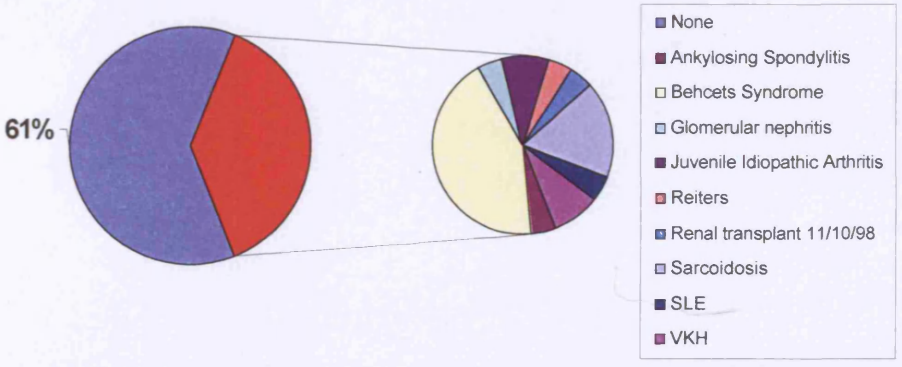


Figure 3.4: Systemic Associations



3.4.1 ANALYSIS I: Effect of Uveitis Diagnosis

Patients with a diagnosis of uveitis (both on and off treatment) had significantly different IFNγ but not IL-2 production compared to healthy controls ('Normals'). IFNγ production was significantly increased in both the CD4+ and CD8+ subsets (p=0.008 and p=0.003 respectively). Figures 3.5-3.7.

Figure 3.5: Statistical Analysis; Normals vs Uveitis Patients

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Mann-Whitney Testing					
Normals vs Uveitis patients	Sig.	0.564	0.398	0.008	0.003

Figure 3.6: CD4 Cells Producing IFNγ; Uveitis Patients vs Normals

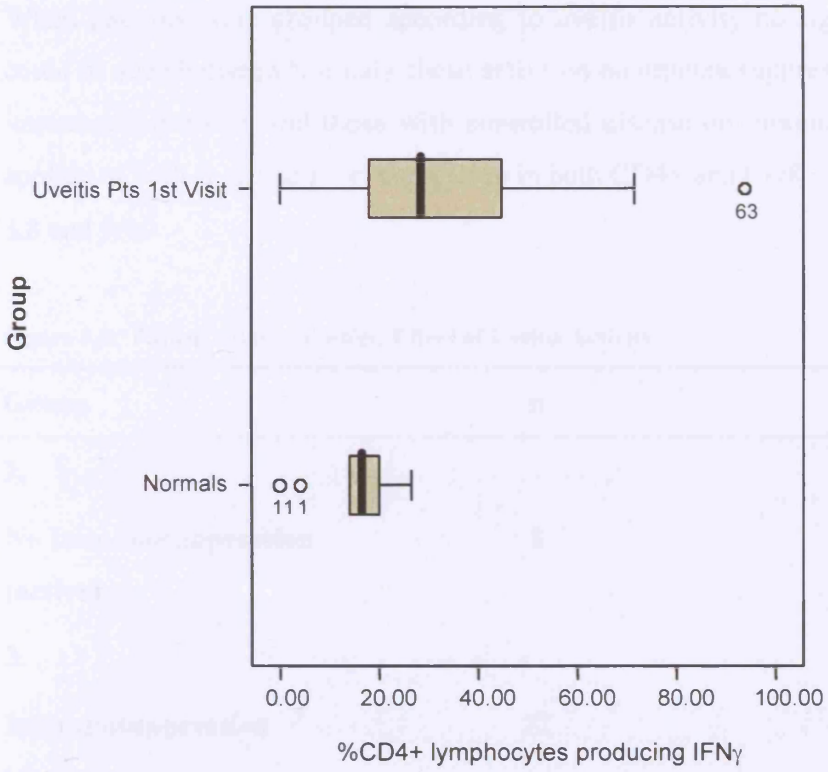
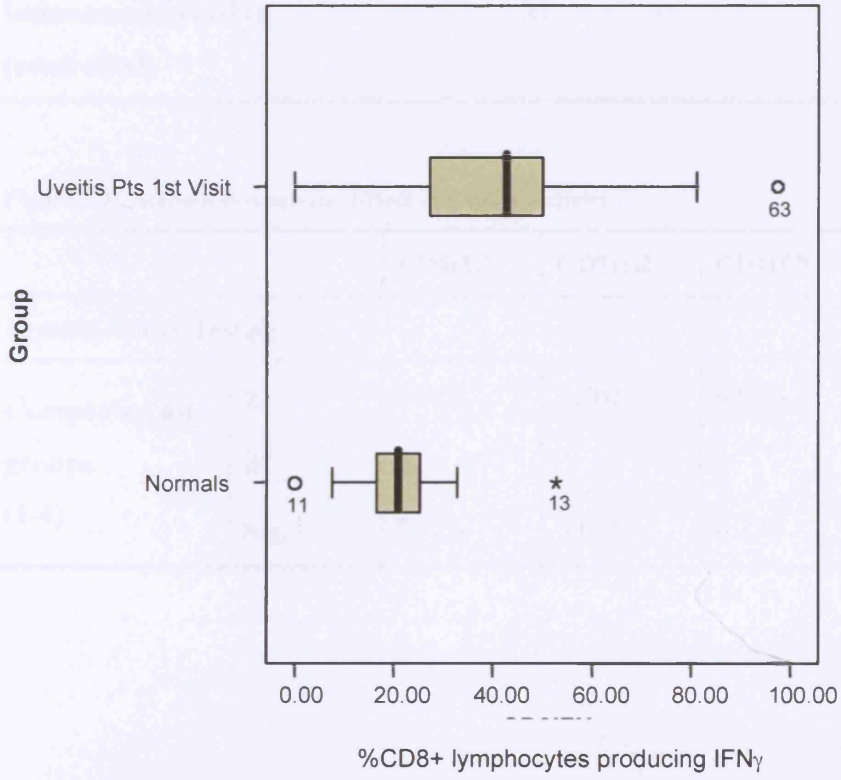


Figure 3.7: CD8 Cells Producing IFNγ; Uveitis Patients vs Normals



3.4.2 ANALYSIS II: Effect of Uveitis Activity

When patients were grouped according to uveitis activity no significant differences could be seen between Normals, those active on no immunosuppression, those active on immunosuppression and those with controlled disease on immunosuppression. This applied to both IL-2 and IFNγ expression in both CD4+ and CD8+ subsets. See Figures 3.8 and 3.9.

Figure 3.8: Patient Characteristics: Effect of Uveitis Activity

Group	n	Age (range)
2.		
No Immunosuppression (active)	8	33 (21-68)
3.		
Immunosuppression (active)	22	41 (16-66)
4.		
Immunosuppression (controlled)	31	50 (22-78)

Figure 3.9: Statistical Analysis; Effect of Uveitis Activity

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-4)	χ^2	6.487	7.263	0.983	1.779
	df	3	3	3	3
	Sig.	0.116	0.066	0.776	0.486

3.4.3 ANALYSIS III: Effect of Associated Systemic Disease

When patients were grouped according to whether they had purely ocular disease or an associated systemic disease no differences were detected in IL-2 or IFNγ expression in both CD4+ and CD8+ subsets.

Figure 3.10: Patient Characteristics; Effect of Associated Systemic Disease

Group	n	Age (range)
2.		
Ocular Syndrome	37	50 (16-78)
3.		
Systemic Disease	24	47 (22-75)

Figure 3.11: Statistical Analysis; Effect of Associated Systemic Disease

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-3)	χ^2	1.841	2.781	7.170	8.567
	df	2	2	2	2
	Sig.	0.398	0.249	0.028	0.014
Mann-Whitney Testing					
1 vs 2	Sig. (Corrected)	N/A	N/A	0.007 (0.021)	0.006 (0.018)
1 vs 3	Sig. (Corrected)	N/A	N/A	0.032 (0.096)	0.008 (0.024)
2 vs 3	Sig. (Corrected)	N/A	N/A	0.724 (1)	0.901 (1)

3.4.4 ANALYSIS IV: Effect of Drug Regime

Kruskall Wallis testing identified changes in IL-2 production by CD4+ cells (p=0.007) and CD8+ cells (p=0.009) when comparing all the subgroups for analysis of drug regime as shown in Figure 3.11. No differences in IFNγ production were seen in this sub-group analysis. Further testing was able to demonstrate a significant fall in IL-2 production by CD4+ cells of patients on a Steroid + CsA regime compared to Normals (p=0.012). A similar fall was seen in IL-2 production by CD8+ cells of patients on a Steroid + CsA regime compared to Normals but this did not achieve significance after correcting for multiple comparisons (p=0.066). None of the other drug regimes were demonstrated to show significant differences in IL-2 production compared to normal controls. See Figures 3.10-3.13.

Further analysis using Kruskal Wallis testing suggested between sub-group differences in IL-2 production by CD4+ and CD8+ cells. See Figure 3.14. Multivariate analysis was not able to identify a significant difference in IL-2 production by CD4+ cells in the different patient groups. In the CD8+ subset a significant difference was seen between CD8+ production of IL-2 by patients on a Steroid + CsA regime and CD8+ production of IL-2 by patients on a Steroid + Aza regime (p=0.02).

Figure 3.12: Patient Characteristics; Effect of Drug Regime

Group	n	Age (range)
2. No Immunosuppression	8	33 (21-68)
3. Steroids Alone	15	41 (22-66)
4. Steroids + CsA	16	40 (16-62)
5. Steroids + Aza	6	57 (49-75)
6. Steroids + MMF	9	54 (31-76)
7. Others	7	61 (49-78)

Figure 3.13: Statistical Analysis 1; Effect of Drug Regime

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-7)	χ^2	17.695	16.953	7.560	9.760
	df	6	6	6	6
	Sig.	0.007	0.009	0.272	0.135
Mann-Whitney Testing					
1 vs 2	Sig.	0.796	0.796	N/A	N/A
	(Corrected)	(1)	(1)		
1 vs 3	Sig.	0.123	0.504	N/A	N/A
	(Corrected)	(0.738)	(1)		
1 vs 4	Sig.	0.002	0.011	N/A	N/A
	(Corrected)	(0.012)	(0.066)		
1 vs 5	Sig.	0.815	0.024	N/A	N/A
	(Corrected)	(1)	(0.144)		
1 vs 6	Sig.	0.949	0.606	N/A	N/A
	(Corrected)	(1)	(1)		
1 vs 7	Sig.	0.087	0.029	N/A	N/A
	(Corrected)	(0.522)	(0.174)		

Figure 3.14: CD4 Cells Producing IL-2; Effect of Drug Regime

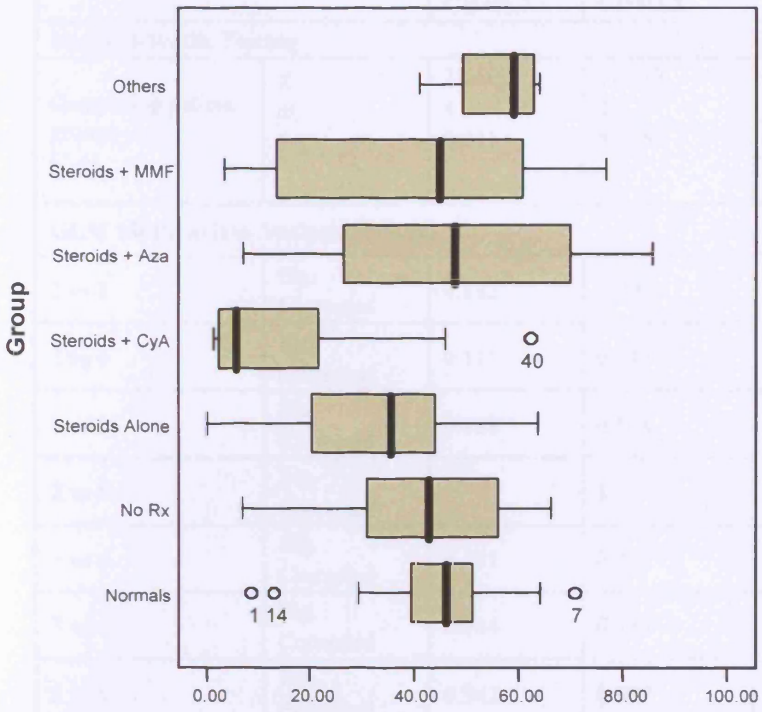


Figure 3.15: CD8 Cells Producing IL-2; Effect of Drug Regime

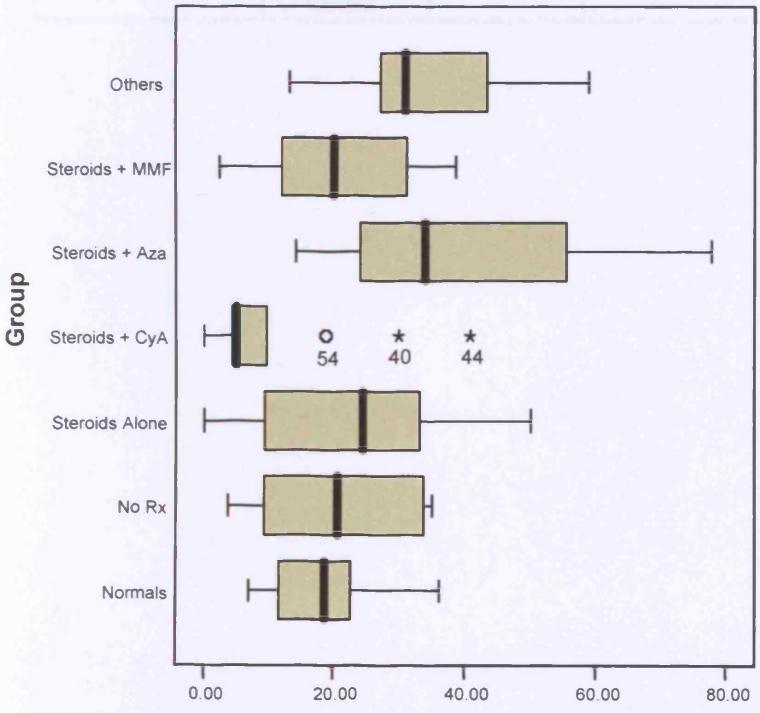


Figure 3.16: Statistical Analysis 2; Effect of Drug Regime

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing patient groups (2-6)	χ^2 df Sig.	10.602 4 0.031	11.117 4 0.025	N/A	N/A
GLM Multivariate Analysis (Tukey)					
2 vs 3	Sig. Corrected	0.885	0.996	N/A	N/A
2 vs 4	Sig. Corrected	0.115	0.543	N/A	N/A
2 vs 5	Sig. Corrected	0.988	0.148	N/A	N/A
2 vs 6	Sig. Corrected	1	1	N/A	N/A
3 vs 4	Sig. Corrected	0.401	0.207	N/A	N/A
3 vs 5	Sig. Corrected	0.644	0.183	N/A	N/A
3 vs 6	Sig. Corrected	0.942	0.997	N/A	N/A
4 vs 5	Sig. Corrected	0.057	0.002	N/A	N/A
4 vs 6	Sig. Corrected	0.164	0.526	N/A	N/A
5 vs 6	Sig. Corrected	0,969	0.154	N/A	N/A

3.4.5 ANALYSIS V: Effect of CsA Dose

Patients on a Steroid + CsA regime were ordered according to CsA dose to assess if the dose of CsA could be correlated with IL-2 production. Figures 3.15 and 3.16 are scatter plots of cytokine expression against CsA dose. Spearman's testing did not reveal any significant correlations.

Figure 3.17: CD4 Cells Producing IL-2; Effect of CsA Dose

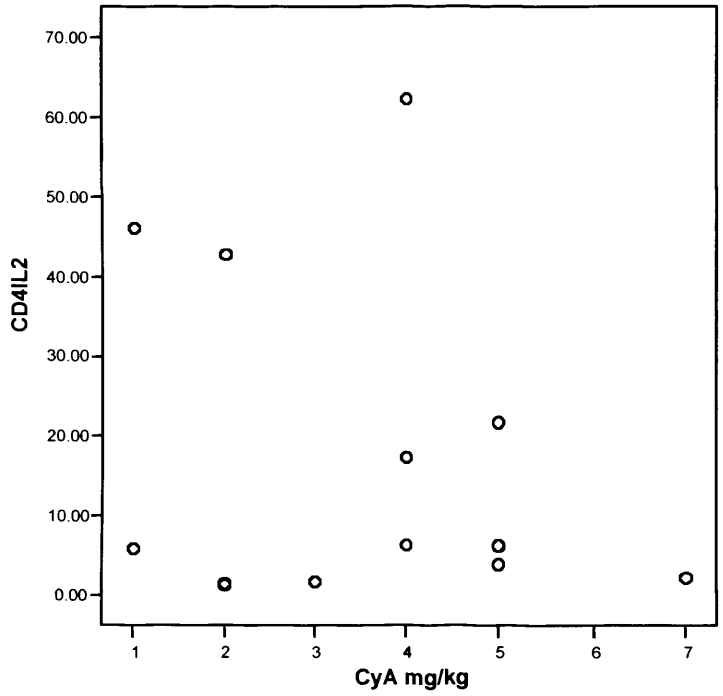
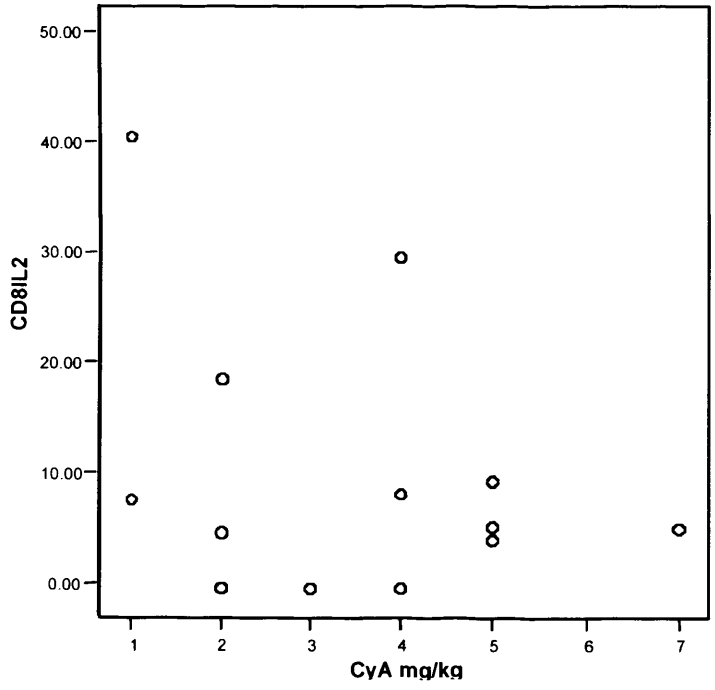


Figure 3.18: CD8 Cells Producing IL-2; Effect of CsA Dose



3.4.6 ANALYSIS VI: CD69 Expression

To assess whether low IL-2 or IFNγ levels were due to problems with activation a number of samples were also tested for CD69. CD69 data was available for 81 out of a total of 120 samples processed.

84% of PMA + ionomycin stimulated samples showed 80% or more CD69 expression (See Figure 3.17). Median CD69 expression of CD3+ cells was 95% (range 2-100). Interestingly the patient with 2% CD69 expression on stimulation had good IL-2 and particularly IFNγ expression despite poor markers of activation. She was being treated with systemic immunosuppression for glomerular nephritis before presentation. Clinically she had an endophthalmitis type picture with a visual acuity of hand movements, an actively inflamed left eye with, flocculant material in the anterior chamber and no fundal view. No microbiological diagnosis was comfirmed.

Kruskall Wallis testing (1 sample per patient; n=38) showed no significant difference in CD69 expression between drug groups (p=0.360).

67 of these samples (83%) had less than 1% of CD3+ cells showing CD69+ expression under non-stimulated conditions. The median of the unstimulated samples showing more than 1% CD69+ expression was 2 (range 1-15). Figure 3.18 describes the patients showing unstimulated CD69+ expression.

Figure 3.19: Stimulated CD3 Cells Expressing CD69

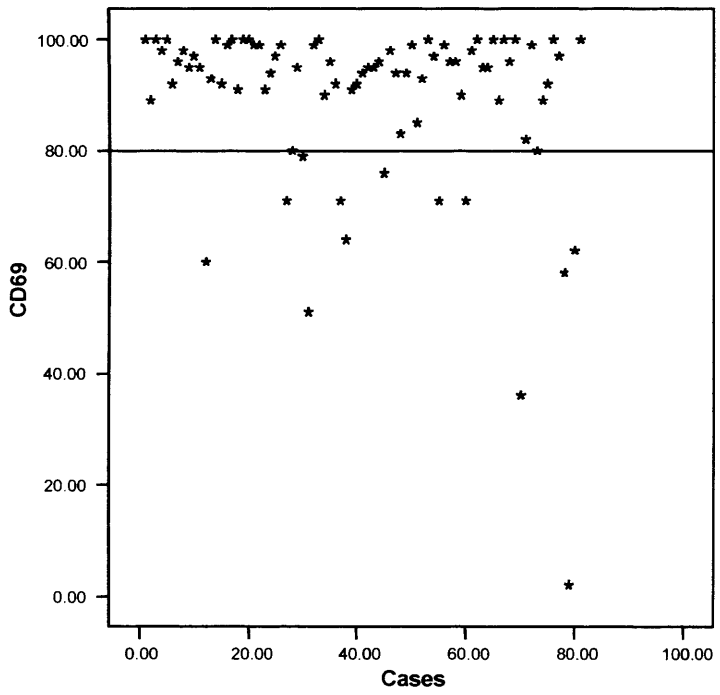


Figure 3.20: Unstimulated CD3 Cells Expressing CD69; Pt Characteristics

Pt. no	Diagnosis	Visit	Visual Acuity	Activity	Drug Regime	CD69 unstim	CD69+ stim
5	VKH	1	6/18:6/24	Active	nil	6	100
7	Idiopathic Intermediate	3	6/12:6/24		10mg Pred	4	100
10	Idiopathic Panuveitis	3	6/18:6/36		30 mg Pred + 300mg CyA	7	94
11	Idiopathic Retinal Vasculitis	2	6/6:6/6		15mg Pred + 300mg CyA	2	94
12	Behcet's Panuveitis	1	6/18:6/36		25mg Pred + 2g MMF	1	100
12	Behcet's Panuveitis	2	6/18:6/36	Active	25mg Pred + 2g MMF	12	71
12	Behcet's Panuveitis	3	6/18:CF		30mg Pred + 2g MMF	3	36
15	JCA	2	6/36:6/36	Active	10mg Pred + 350mg CyA	2	95
23	Behcet's Panuveitis	2	6/9:6/9		5mg Pred + 50mg Aza	15	97
26	Idiopathic Panuveitis	3	6/60:6/18		25mg Pred + 200mg CyA + 150mg Aza	2	58
30	Birdshot C-R	4	6/9:HM		5mg Pred	2	99
47	BARN	2	NPL:HM		20mg Pred + 150mg Aza	1	90
47	BARN	3	NPL:HM		15mg Pred + 150mg Aza	2	96
59	Scleritis	1	6/6:6/6	Active	50mg Pred + 150mg Aza	2	93

3.4.7 ANALYSIS VII: Repeat Samples

A total of 120 serum samples were taken from the uveitis patients at different clinic visits and at different levels of immunosuppression. Figure 3.19 shows the immunosuppression regimes at the time of sampling. 32 patients provided repeat samples at multiple visits (see Figure 20). The median time between first and second visits was 49 days (range 0-151). The majority of patients showed a reduction in IL-2 and IFNγ expression at the second visit compared to the first. This was seen to be most significant for IFNγ expression by CD8+ cells (p=0.023 See Figure 3.21).

Figure 3.21: Drug Regime at Sample Points

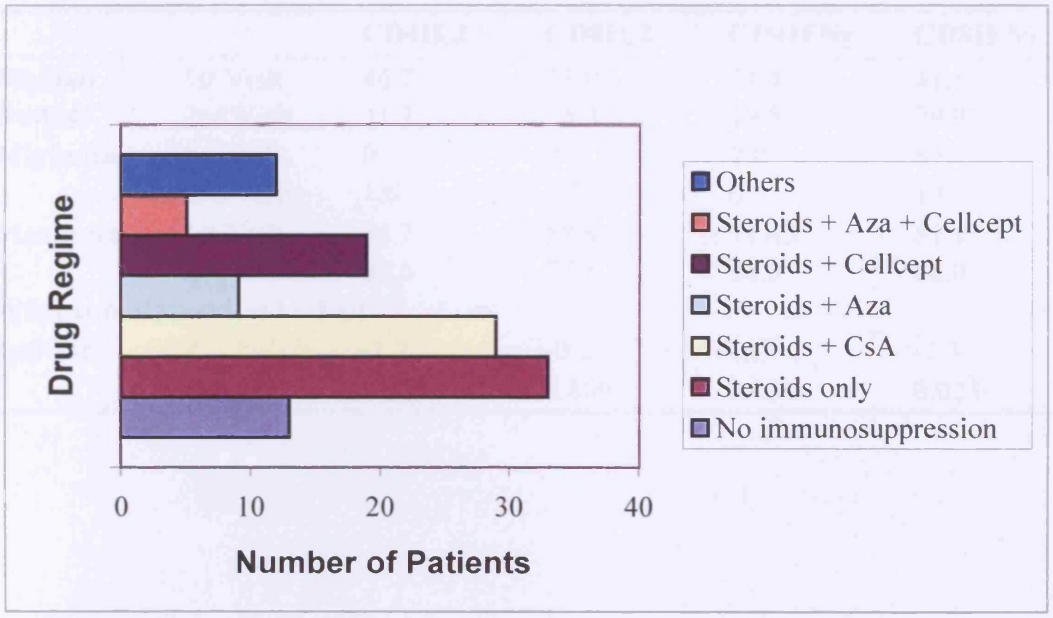


Figure 3.22: Frequency of Uveitis Visits

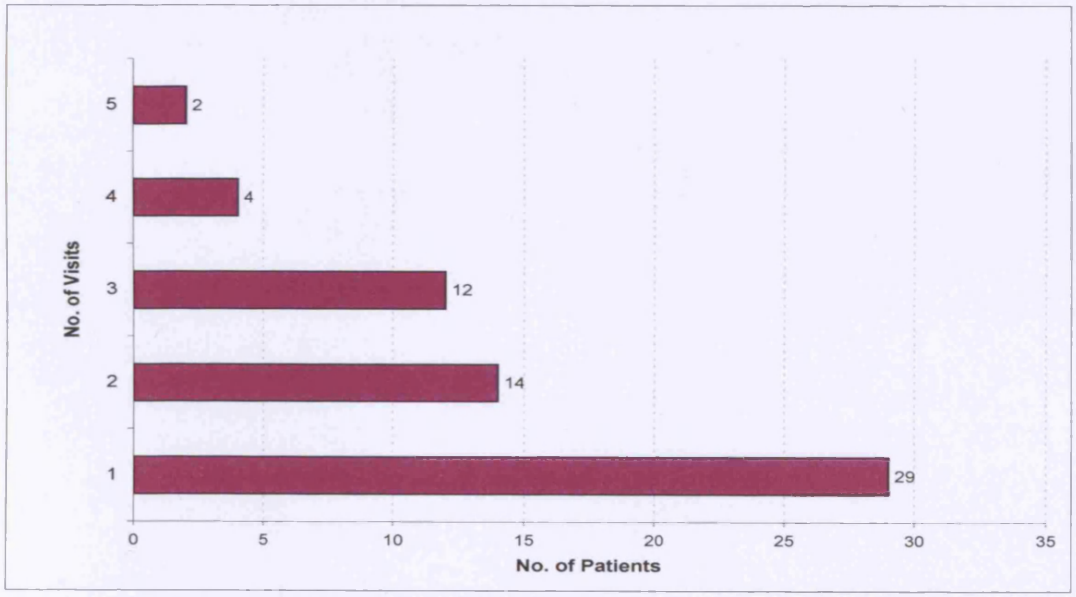


Figure 3.23: Multiple Visits; Comparing 1st and 2nd Visits

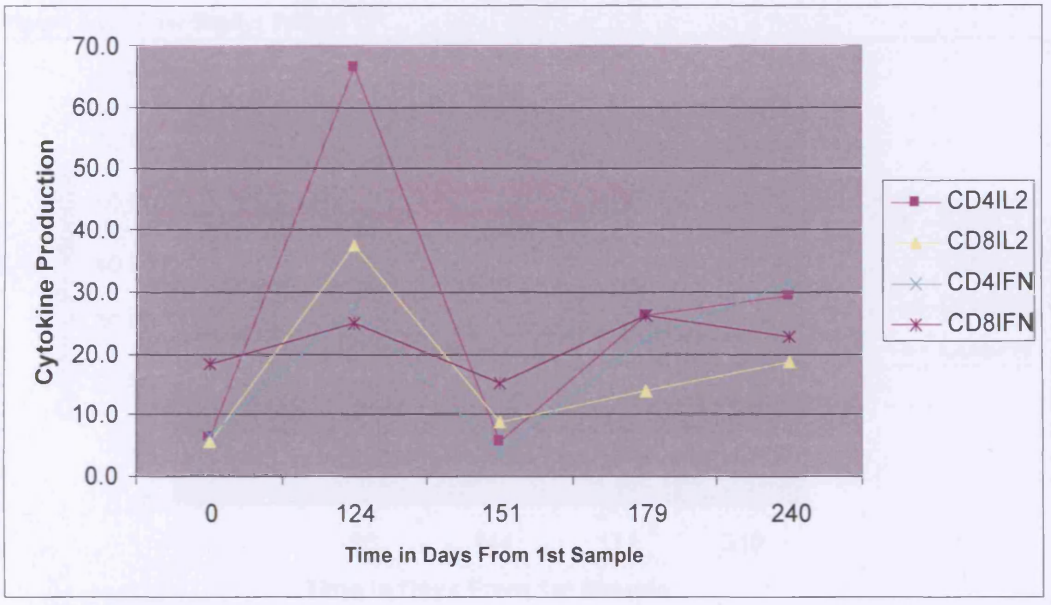
		CD4IL2	CD8IL2	CD4IFNγ	CD8IFNγ
Median (range)	1st Visit	46.7	23.0	24.4	41.1
	2nd Visit	31.7	26.3	19.5	29.0
Minimum	1st Visit	0	0	2.8	6.9
	2nd Visit	1.6	1.7	0	1.6
Maximum	1st Visit	84.2	58.8	71.43	81.1
	2nd Visit	85.6	77.8	50.0	68.0
Wilcoxon Signed Ranks Test					
2nd-1st	Z	-1.7	-0.2	-1.7	-2.3
	Sig.	0.082	0.809	0.086	0.023

3.4.7.1 Case Studies

3.4.7.1.1 Patient 19

Patient 19 was a 32 year old woman with Behcet's disease. Figure 3.22 charts her clinical progress, immunosuppression regime and IL-2 and IFNγ production over a period of 8 months. Her initial activation in the left eye appears to be associated with an increase in both IL-2 and IFNγ production. Although cytokine production is then effectively reduced by an increase in immunosuppression the patient reactivates in the other eye. Clinical control of the inflammation occurs despite a small increase in IL-2 and IFNγ production by CD4+ cells.

Figure 3.24: Case Study; Patient 19

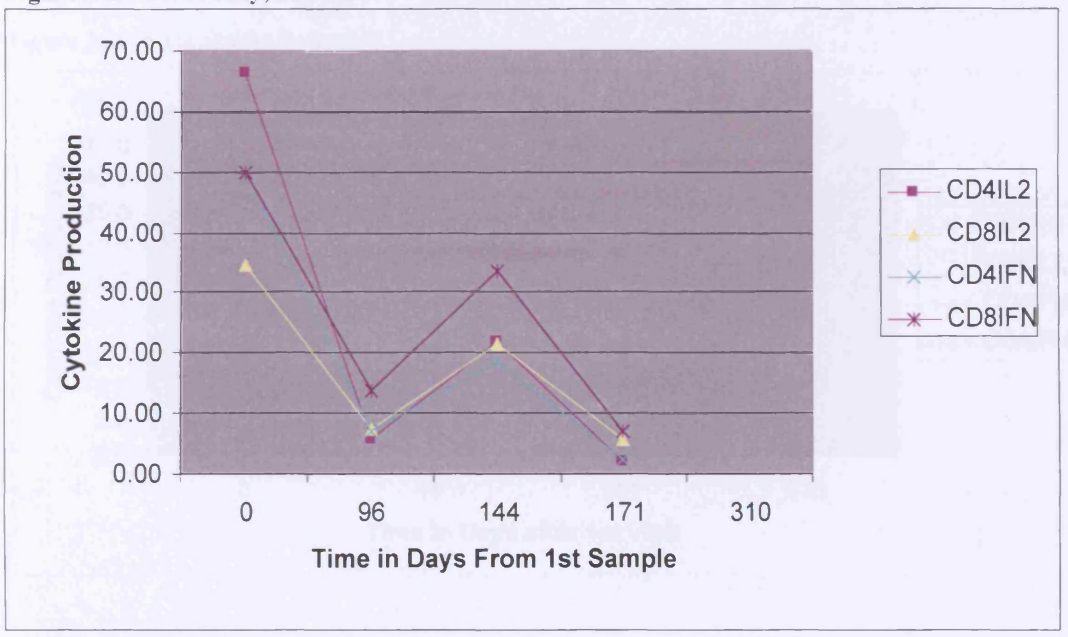


	0	124	151	179	240
		ACTIVE	ACTIVE		
Visual Acuity	CF:6/24	CF:6/60	CF:CF	CF:CF	CF:HM
Drug Regime	10mg Pred 300mg CyA	10mg Pred 50mg CyA	35mg Pred 100mg CyA	25mg Pred 100mg CyA	17.5mg Pred 100mg CyA

3.4.7.1.2 Patient 15

Patient 15 was a 32 year old man with juvenile idiopathic arthritis. Figure 3.23 follows his clinical progress, drug regime and IL-2 and IFNγ production over a period of 5 months. Samples taken at 10 months were analyzed as 'missing' because of insufficient information. At his first visit he was on no systemic immunosuppression and was noted to have recent onset cystoid macular oedema. This was associated with both high IL-2 and IFNγ production. Treatment with steroids and CsA can be seen to have effectively reduced cytokine expression although, similar to patient 19, control of type 1 cytokine expression was not associated with clinical control of the inflammation. In fact cytokine expression had risen slightly at the third visit despite control of inflammation.

Figure 3.25: Case Study; Patient 15

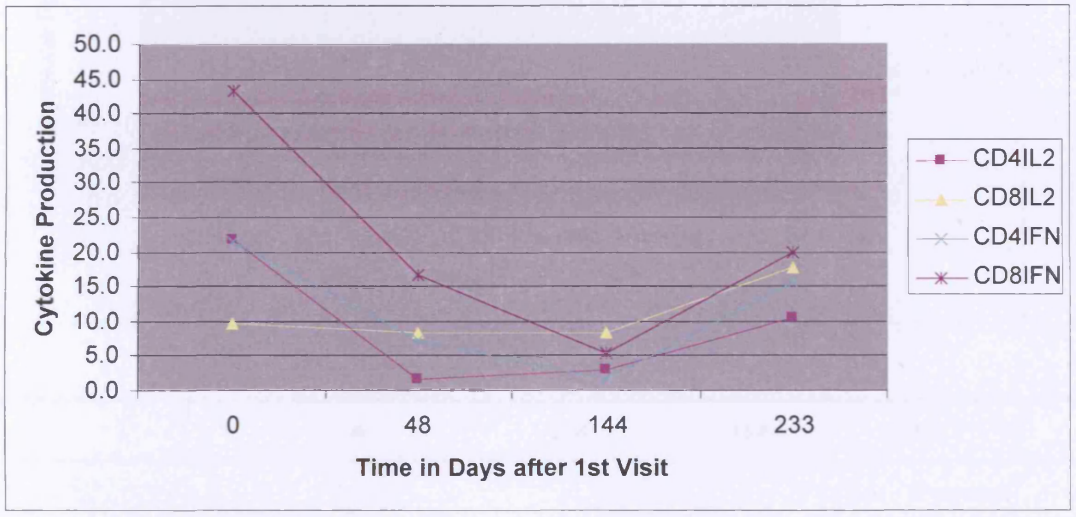


Time in Days	0	96	144	171	310
	ACTIVE	ACTIVE			R post capsulectomy + vity day 239
Visual Acuity	6/60:CF	6/36:6/36	6/36:6/60	CF:CF	6/12:HM
Drug Regime	0	10mg Pred 350mg CyA	10mg Pred 250mg CyA	8mg Pred 350mg CyA	12mg Pred 350mg CyA

3.4.7.1.3 Patient 11

Patient 11 was a 26 year old Caucasian woman with idiopathic retinal vasculitis. Figure 3.24 follows her over a period of 8 months. At time point 0 she was clinically active with vasculitis and cystoid macular oedema. Testing showed elevated levels of IL-2 and IFNγ production with the IFNγ production being particularly high in the CD8+ subset. This is despite treatment with 35mg of prednisolone and 500mg of CsA. Clinically there was a good response to treatment and the immunosuppression was tapered. Flow cytometry showed a reduction in cytokine production compared to the initial high response although there was a small increase noted at the 4th visit when immunosuppression had been reduced to 5 mg prednisolone and 150mg CsA. At this time ocular appearances were clinically normal.

Figure 3.26: Case Study; Patient 11

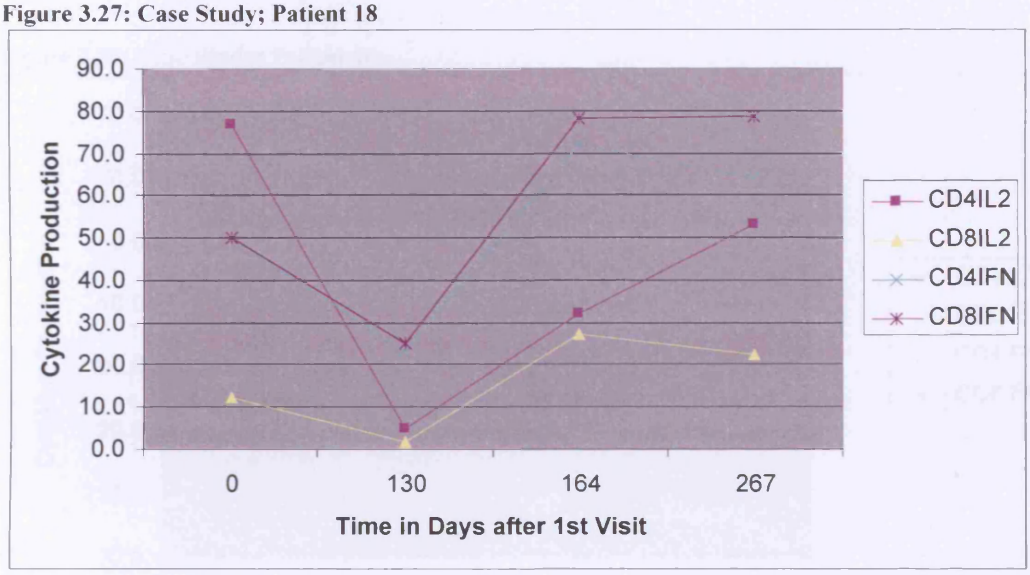


	0	48	144	233
	ACTIVE			
Visual Acuity	6/6:6/6	6/6:6/6	6/6:6/6	6/6:6/6
Drug Regime	35mg Pred	15mg Pred	7.5mg Pred	5mg Pred
	500mg CyA	300mg CyA	300mg CyA	150mg CyA

3.4.7.1.4 Patient 18

Patient 18 was a 76 year old woman with panuveitis and a diagnosis of sarcoidosis. Figure 3.25 follows her over a period of 8 months. At none of the visits where cytokine activity was measured was this woman active although she did have recurrences requiring an increase in immunosuppression over this time period. Her steroid dose was increased to 40mg prednisolone at the second visit and this can be seen to be associated with dramatically reduced IL-2 and IFNγ production.

Figure 3.27: Case Study; Patient 18

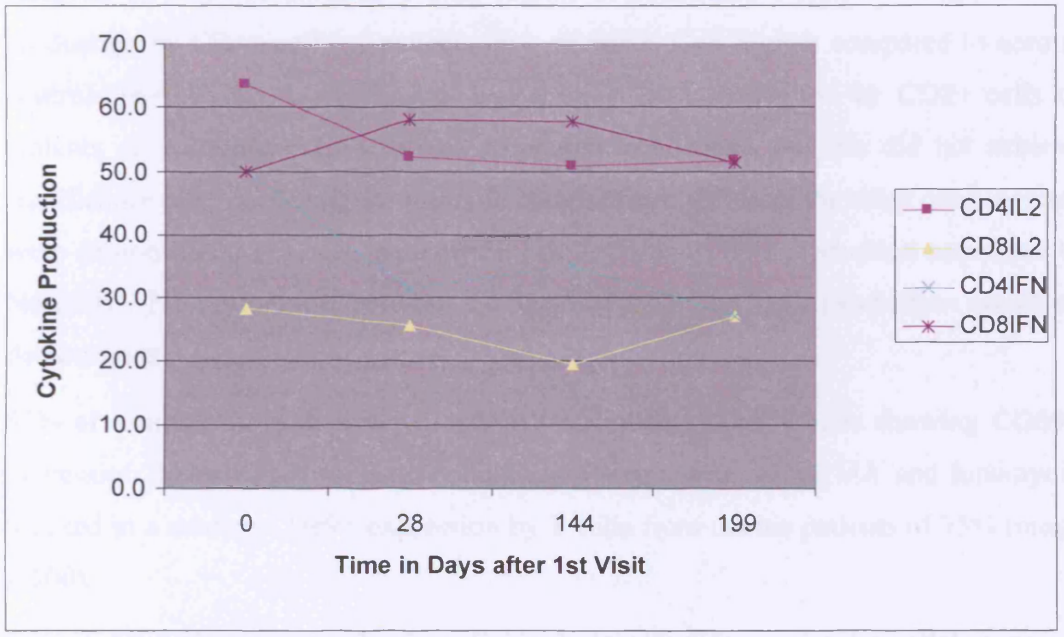


	0	130	164	267
				R cataract
Visual Acuity	6/18:6/24	6/18:6/60	6/12:6/36	6/60:CF
Drug Regime	15mg Pred	40mg Pred	20mg Pred	20mg Pred
	2g MMF	2g MMF	2g MMF	2g MMF

3.4.7.1.5 Patient 30

Patient 30 was a 50 year old man with Birdshot chorioretinitis. Figure 3.26 follows him over a period of 3 months. He was active at his first visit and prescribed oral prednisolone. Unfortunately he did not comply with treatment and was active when seen taking no prednisolone 1 month later. Treatment with high dose steroids was reinitiated and IL-2 and IFNγ production remained remarkably constant despite control of the intraocular inflammation.

Figure 3.28: Case Study; Patient 30



	0	28	144	199
	ACTIVE	ACTIVE		
Visual Acuity	6/6:HM	6/6:HM	6/9:HM	6/9:HM
Drug Regime	60mg Pred	0mg Pred	7.5mg Pred	5mg Pred

3.5 SUMMARY

Autoimmune uveitis patients had significantly different IFNγ but not IL-2 production compared to normal controls. IFNγ production was significantly increased in both the CD4+ and the CD8+ subsets (p=0.008 and p=0.003 respectively).

No significant differences in type 1 cytokine production by both CD4+ and CD8+ cells could be seen when patients where analyzed according to uveitis activity or according to systemic versus purely ocular disease.

Analysis of results according to drug regime demonstrated a significant fall in IL-2 production by CD4+ cells of patients on a steroid + CsA regime compared to normal controls (p=0.012). A similar fall was seen in IL-2 production by CD8+ cells of patients on a steroid + CsA regime compared to Normals but this did not achieve significance after correcting for multiple comparisons. None of the other drug regimes were demonstrated to result in significant differences in IL-2 production compared to Normals. No correlation between the dose of CsA and IL-2 production could be demonstrated.

83% of samples from uveitis patients had less than 1% of T cells showing CD69+ expression under non-stimulated conditions. Stimulation with PMA and ionomycin resulted in a median CD69+ expression by T cells from uveitis patients of 95% (range 2-100).

Examination of repeat samples in individual patients did not reveal parallels between percentage expression of cytokine and the clinical course of disease.

3.6 DISCUSSION

Analysis to investigate whether uveitis patients have different Th1 cytokine expression compared to that of Normals found that IFNγ production was significantly increased in both CD4+ and CD8+ T cells of patients. This is consistent with reports of Th1 cytokine profiles in active autoimmune disease(254). The finding supports that of Lacomba and colleagues who used ELISA techniques to demonstrate elevated IFNγ production in both the serum and aqueous of 23 patients with various uveitides(341). Imai and colleagues demonstrated a similar finding using mRNA techniques and flow cytometry in 9 patients with Vogt-Koyanagi-Harada syndrome although they report elevated IFNγ and IL-2 production in both stimulated and unstimulated conditions(324). Ooi et al

have demonstrated significantly elevated IFNγ in pooled aqueous from 36 patients with active uveitis compared to cataract controls(379). Skurkovich and Skurkovich have used intramuscular anti-IFNγ antibodies in the treatment of various autoimmune diseases and reported good results and tolerability in preliminary clinical investigations(380, 381). Interestingly they also reported that topical anti-IFNγ antibodies were helpful in reducing inflammation in 13 eyes undergoing rejection following penetrating keratoplasty(382). A difference between IL-2 expression in patients and Normals was not seen although the effect may have been masked in the IL-2 subsets by the effect of drugs.

No significant difference in IL-2 and IFNγ production of patients with systemic disease compared to those with purely ocular disease was demonstrated suggesting the importance of peripheral activation even for organ specific disease. Further analysis to find the effect of clinical uveitis activity was unable to detect a significant difference between Normals, those active on no immunosuppression, those active on immunosuppression and those with controlled disease on immunosuppression. This was a surprising result and was true for both for IL-2 and IFNγ expression. The effect of the drugs would be expected to result in a difference and in contrast Ohno and colleagues reported elevated IFNγ production in BD patients that varied with the stage of disease(325).

Our failure to demonstrate a difference may have been due to difficulties in the classification of patients into these groups. Clinical activity in uveitis is notoriously difficult to assess particularly in the setting of chronic disease(288, 383-386). In this study patients were assessed by the clinic doctor at their usual clinic appointment resulting in inter-observer variation. This was minimised by having a study proforma that was filled out for each patient but could have been overcome by having each study patient examined by the same observer. LogMar visual acuity testing would provide better quantitative information on visual acuity(387). Laser flare readings(388-390) provide objective measurements which can be useful as an adjunct to clinical examination when deciding on activity, particularly in the setting of chronic disease. Similarly Optical Coherence Tomography is now widely used in uveitis clinics to aid the diagnosis of macular oedema even in the context of hazy media. Both these tests were not widely used at the time of this study. Photographic documentation would have improved reliability by reducing subjectivity.

Samples taken at fixed times along the time course of disease progression would be easier to interpret and if each patient was sampled before starting therapy this would have provided the ideal control group for assessment of the effect of therapy on cytokine production. This, however, was difficult to achieve in the setting of this tertiary referral clinic as patients presented at varying points in the progression of their disease and on varying medications. Even after presentation the chronic or relapsing, remitting nature of the disease entities resulted in very different follow up profiles. Where possible samples were taken from patients before starting treatment to provide a control group for comparisons (No immunosuppression, active) but this group was small (n=8).

A major limitation of these studies is that they have been performed in a heterogenous group of uveitis patients. Each uveitis diagnostic category will have a typical natural history and response to immunosuppression but this varies markedly between and sometimes within categories. Treatment must always be adjusted to an individual's response so drug regimes are in a constant and often unpredictable state of flux. Because most of the uveitis conditions requiring systemic immunosuppression are uncommon or rare identifying a more homogenous study group would lead to much reduced numbers and an even further reduction in power to assess the effect of drug dose on cytokine production.

In 1998 Van den Berg and colleagues published work in which they were able to use flow cytometry to quantify the level of immunosuppression in transplant patients(282). They were able to show a negative correlation between the percentage of CD4+ T cells producing IL-2 and CsA levels and suggested that prospective studies should be performed to determine whether this method is useful for clinical monitoring.

Sub-group analysis to investigate the effect of drug regime on Th1 expression showed no differences in IFNγ expression as would be expected considering that none of the drugs target IFNγ expression specifically. Testing for sub-group differences in IL-2 production revealed inhibition of IL-2 production in patients on Steroid + CsA regimes compared to Normals. This inhibition reached statistical significance in the CD4+ but not the CD8+ subset. The lack of significance in the CD8+ subset is likely to be due to the conservative adjustment for multiple comparisons and would require further investigation to confirm or refute. In view of what is known about the mechanisms of actions of steroids some changes in IL-2 and perhaps IFNγ production for patients

compared to Normals may have been expected. This was not demonstrated statistically and comparisons of medians does not suggest a measurable change in IL-2 production compared to Normals (See Charts III(i&ii)). Likewise no changes in IL-2 or IFNγ production of patients on Aza or MMF regimes were seen compared to Normals but this is in keeping with what is known about the mechanism of action of these drugs(361, 362, 391). Kilmartin et al also found no significant changes between control groups and uveitis patients on MMF for IL-2 and IFNγ intracellular cytokine expression(345).

The effect of CsA dose was analyzed to assess whether intracellular cytokine analysis of IL-2 could be used as a marker of the degree of immunosuppression. Charts V(i&ii) demonstrate that most patients on Steroid + CsA had effective suppression of IL-2 even at lower doses. A direct relationship between CsA dose and IL-2 expression was not seen.

CD69 expression by stimulated T cells was found to be high in the vast majority of samples. This is in contrast to published reports that CD69 expression is completely blocked by brefeldin A in mice(392). Kilmartin and colleagues were also able to measure CD69 expression in the presence of brefeldin A(345). They reported CD69 expression in unstimulated CD4+ cells from 10 uveitis patients that paralleled disease activity and was reduced by MMF therapy. Their results were not replicated in this study. Resting CD3+ cells were found to express CD69 at very low levels median 0 (range 0-15%). These differences may be due to differences in protocols or perhaps to low grade antigenic contamination of unstimulated wells.

Repeated testing in individual patients to assess the effect of different doses of immunosuppression and different levels of activity showed that elevated IL-2 and IFNγ expression is associated with uveitis although parallels between the percentage expression and the clinical course of disease were not demonstrated.

Despite its limitations, as discussed above, this study does not support the use of flow cytometric assessment of intracellular cytokines to quantify the level of immunosuppression and thus aid clinical management in patients receiving systemic immunotherapy for ocular indications.

CHAPTER 4

ASSESSMENT OF CYTOKINE PRODUCTION

IN

HIV POSITIVE PATIENTS

4.1 INTRODUCTION

4.1.1 Pathology of HIV Infection

4.1.1.1 Classification of Viruses

HIV-1 and -2 are enveloped RNA viruses belonging to the lentivirus subgroup of the retrovirus family(393). Lentiviruses are characterized by cytopathicity in vitro, lack of oncogenicity, establishment of chronic infections and slow rates of development of disease. HIV-1 has a worldwide distribution and is thought to have developed from cross-species transmission from chimpanzees. HIV-2 in contrast is more prevalent in Africa and is thought to have developed from cross-species transmission from another primate, the sooty mangabey. HIV-1 has been further divided into subgroups and subtypes. Transmission of virus is by direct contact with infected bodily fluids such as blood and semen. HIV targets the CD4+ T-cell population leading to immune activation followed by eventual depletion of CD4 cells and development of AIDS.

4.1.1.2 Viral Replication

Viral replication is initiated by attachment of the virus to a target cell through the interaction of the viral envelope glycoprotein, gp120, with the CD4 receptor. The primary cellular targets for HIV-1 infection in vivo are CD4+ T cells and macrophages. CD4+ binding induces conformational changes in gp120 that enables binding to the cellular co-receptor molecules CXCR4 and CCR5. Binding to these co-receptors allows fusion of the virus with the host cell and the natural ligands can block the infectivity of HIV-1. The natural ligand for CXCR4 is stromal cell-derived factor 1 and CCR5 naturally binds to the β-chemokines. Following fusion, synthesis of viral cDNA by reverse transcription can be completed in the target cell cytoplasm. The cDNA is then translocated to the nucleus where it is integrated into the host cell chromosome by the virally encoded integrase in concert with host cell DNA repair enzymes to form a provirus. HIV-1 can infect both resting and activated CD4+ T cells but integration of HIV-1 DNA into the nucleus probably does not occur unless the T cell is antigen activated. In resting T cells reverse transcribed HIV-1 genomes can stay in the cytoplasm for days to weeks awaiting activation before they become non-functional. Once the DNA is integrated, activation of HIV transcription and gene expression is dependent on the activity of both cellular and viral factors. Structural and enzymatic proteins are produced and the virion assembles at the plasma membrane. Completed virions incorporate a number of cellular proteins including major histocompatability

antigens and adhesion molecules as well as the virally encoded proteins. Infectious virions then bud through the plasma membrane. Productively infected cells generally die within a few days from cytopathic effects but some survive long enough to revert to a resting memory state and thus establish a stable latent reservoir. Upon subsequent exposure to antigen these cells will become activated and release infectious virions. Approximately 10^{10} virions are generated daily.

4.1.1.3 Time Course of Infection

There are 3 phases in the natural history of HIV-1 infection: primary infection, progressive immune depletion and AIDS.

4.1.1.3.1 Primary Infection

During the primary or acute phase virus present in the infecting inoculum replicates in the host and produces a viremia. 50-70% of individuals infected with HIV-1 are symptomatic during this acute phase with an infectious mononucleosis type syndrome appearing 1 to 12 weeks after exposure. A vigorous antiviral immune response is generated which eventually leads to a dramatic reduction in viremia. Virus specific CD8+ T cells appear early and probably represent a critical host factor in the control of acute HIV-1 infection. Antibodies to HIV-1 are initially absent and seroconversion usually occurs within a few weeks of the onset of the acute illness. Nearly all infected individuals develop antibody responses to several of the protein products of the HIV-1 genome. Unfortunately most of these are non-neutralizing. The CD4+ T cell count diminishes during symptomatic primary HIV-1 infection due to virus-induced CD4+ T cell depletion and sequestration of circulating CD4+ T cells in lymphoid organs. After the acute illness resolves CD4+ T cells counts tend to rise again but usually not to pre-infection levels.

4.1.1.3.2 Progressive Immune Depletion

Primary infection is followed by a long period of often asymptomatic infection. Clinically this is a time of latency but the virus continues to replicate reaching a steady state, the level of which determines the rate of disease progression. There is a gradual loss of CD4+ T cells. Both viral load (HIV-1 RNA level) and CD4 count are used when making decisions about when to initiate therapy. There is huge individual variation in the length of the asymptomatic period with some progressing to AIDS within 2 years and other long-term non-progressors maintaining their CD4+ count for over 20 years. Vigorous virus specific CD8+ T cell responses are seen in many long-term non-

progressors and the frequency of HIV-1 specific CD8+ T cells has been shown in some studies to decline as the clinical and immunological status of the patient deteriorates. Rates of disease progression are affected by viral factors (sequence variation e.g. mutations in *env* leading to a change in the pattern of chemokine receptor use), host factors (e.g. polymorphisms in chemokine receptor or chemokine genes) and environmental factors (e.g. concurrent infections). Increased rates of apoptosis lead to loss of CD4+ cells. In addition to the depletion of CD4+ T cells that occurs during the course of infection there are qualitative defects in the function of the surviving CD4+ T cells.

4.1.1.3.3 AIDS

Acquired immunodeficiency syndrome is characterized by low or very low CD4 counts. The median time from acquisition of HIV-1 infection to AIDS is approximately 8-10 years. Wasting, thrombocytopenia dementia and neuropathy can occur as a direct consequence of HIV infection. Immunodeficiency leaves affected individuals vulnerable to opportunistic infections and malignancies although these are rare in patients with CD4 counts above 500 cell/mm^3. As the CD4 count drops below 500 cells/mm^3 patients may begin to suffer from oral candidiasis, pneumococcal infection and various skin disorders. The risk of more serious potentially life-threatening opportunistic infections such as *P. jerovici* pneumonia, *Candida* oesophagitis, *Toxoplasma* encephalitis and cryptococcal meningitis increases significantly as the CD4 count falls below 200 cells/mm^3. A CD4 count of less than 200/mm^3 is considered diagnostic of AIDS according to the Centres for Disease Control and Prevention case definition. At CD4 counts under 50/mm^3 patients are at increased risk for the occurrence of infections such as cytomegalovirus retinitis. The HIV pandemic continues worldwide with the World Health Organization estimating that in 2003 37.8 million people were living with the virus worldwide and in that year 2.9 million people died of AIDS.

4.1.2 Effect of Infection on Cytokine Profile

T cell-mediated immune responses are known to be important in the pathogenesis of HIV infection. It has been proposed that a shift from type 1 to type 2 cytokine responses is important in disease progression to AIDS(394, 395). Others have found no evidence for such a shift(396-398). Studies undertaken to investigate the nature of the cytokine responses in HIV have been confusing in their results. The variation in data

may be explained by differences in technique, cell stimulation protocols, cell population or patient population under study(271, 395). In addition T cells are influenced by their environment and in vivo cytokines may be produced by other cells such as monocytes, NK cells or B cells making studies on T cell clones potentially misleading.

Studies using ELISA techniques(263, 397, 399-402) may not accurately reflect the contribution of each cell type as bulk cytokine levels are measured in cell culture supernatants or in serum or plasma samples. Intracellular cytokine staining with flow cytometry is recognized as a method of reliably quantifying lymphocyte cytokine responses at a single cell level (274, 282, 403, 404). Because responses are seen at the single cell level this technique is invaluable in situations such as HIV infection where there may be very low absolute numbers of some cell types. Whole blood techniques ensure that the cellular environment is preserved as far as possible. Studies assessing cytokine genes by reverse transcriptase polymerase chain reaction under non-stimulated conditions attempt to avoid this problem but are limited by the fact that translation into functional proteins may not take place(394, 405-407).

In untreated, HIV infected, individuals reduced IL-2 production with reduced CD4 count appears to be a consistent finding in HIV-disease progression(397). Previous studies have, however, demonstrated both impaired(399, 408-410) (394, 399) and enhanced (397, 400, 405) (403, 405, 411, 412)IFNγ responses. Ullum and colleagues performed ELISA on whole blood supernatants and demonstrated an increase in total IFNγ in patients with HIV and a decrease in patients with AIDS(400). They state that IFNγ production correlated with CD8 count. FACS analysis following intracellular cytokine staining of stimulated T cells supports the conclusion that untreated HIV infected individuals have increased percentages of lymphocytes, particularly of the CD8+ subset, able to produce IFNγ(403, 411, 412).

4.1.3 Treatment of HIV Infection

Since 1996 a number of agents have been used in combination for the treatment of HIV infection. At the time of this study patients were treated with combination anti-retroviral therapy (ART) including nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and protease inhibitors. Inhibitors of HIV fusion have more recently become available. Treatment of HIV infection aims to achieve prolonged suppression of viral replication. When potent combination therapy is successfully administered levels of HIV RNA in plasma and infected cells in lymphoid

tissue rapidly decrease. The rapid first-phase clearance is due to the death of infected activated CD4+ T cells and the prevention of new infections. The second phase clearance rate is slower and more variable. This is attributed to clearance of infected macrophages or chronically infected CD4+ T cells with lower rates of cell death. Clearance of virions bound to dendritic cells in the germinal centres of lymph nodes may also contribute to this second phase. Failure to reduce plasma HIV RNA levels to below the limit of detection of the currently available assays (50 copies/ml) indicates inadequate suppression and a risk for the development of resistant virus. However, even successfully treated individuals do not have complete suppression of virus replication. Latently infected CD4+ T cells may survive for years, archiving virus, often drug resistant, that can re-emerge and replicate after the withdrawal of chemotherapy. High rates of viral turnover allow for the emergence of drug resistant viral mutants. In addition treatment is often limited by cost, drug related toxicities and compliance issues. Despite these considerations treatment with combination antiretroviral therapy (ART) has led to dramatic improvements in HIV-related morbidity and mortality(413, 414).

Suppression of viral replication with therapy allows CD4+ cell numbers to increase and the magnitude of this increase is proportional to the steady-state HIV RNA levels. Therapy results in the rapid redistribution of CD45RO+ memory T cells from the lymphoid tissue(415, 416) back into the circulation and in the slower production of new CD45RA naïve cells. Accompanying this increase in cell numbers is some restoration of immune function. Both CD4+ and CD8+ T cell responses to recall antigens are regenerated leading to a restored ability to deal with opportunistic agents. Primary and secondary prophylaxis for agents such as *Pneumocystis*, *Toxoplasma*, cytomegalovirus, *M. avium* complex, *Cryptococcus* and *Candida* can often be withdrawn(417).

4.1.4 Postulated Effects of Treatment on Cytokine Profile

Clerici and colleagues report that ELISA and mRNA cytokine analysis of resting PBMCs from 2 combination ART treated patients demonstrated reduced IFNγ expression and raised IL-10 expression compared to ART naïve patients(418). In this study they found that IL-2, IL-12 and IFNγ production was robust in ART naïve patients. All patients had less than 500 copies HIV RNA/ml. Martinon and colleagues found a similar result for total resting PBMCs in their 16 patients although the reduction in IFNγ production was not statistically significant(419). Imami and colleagues, in

contrast, found little expression of IFNγ, IL-2, IL-4 or IL-10 mRNA in resting PBMCs of 9 ART naïve individuals using RT-PCR(407). After initiation of combination ART they describe a continuous increase in IFNγ and IL-2 mRNA with successful reduction in viral load and increase in CD4+ T cell counts. Type-2 cytokine specific mRNA expression fell to undetectable levels. This finding is supported by that of Sondergaard and colleagues who report an increase in IFNγ in supernatants tested by ELISA in 12 patients following the initiation of ART(402). FACS analysis of intracellular cytokines in 15 HIV patients in whom protease inhibitors were added to nucleoside analogue regimes demonstrated a statistically significant increase in the proportion of CD4+ cells producing IL-2 to levels similar to those of HIV seronegative controls after 12 months of treatment(420). A similar increase in IL-2 producing CD8+ cells was seen but this did not reach statistical significance in this study. No differences in the proportion of CD4+ or CD8+ cells producing IFNγ could be detected during this time of follow up and the proportion of IL-4 producing CD4+ cells remained <5% in all patients prior to and during treatment with additional protease inhibitors.

Immunological recovery following combination ART is variable with some patients experiencing sustained rises in CD4 cell numbers whilst in other patients CD4 cell counts remain static or fall despite suppression of HIV. Furthermore, persistent functional T cell defects have been shown to occur even in the context of undetectable peripheral blood HIV viral loads and where there has been a rise in total CD4 count(421-423). Lederman et al found many defects in immune function after ART in a large cohort of patients followed for 3 years(424). Interestingly, despite these functional defects very few opportunistic infections occurred during follow up. Song and colleagues report on CMV retinitis patients who had immune restoration on combination ART allowing them to stop CMV prophylaxis but who later lost this protective mechanism when the CD4 count dropped below 50 cells/mm^3(425). They noted that the HIV viral load did not appear to predict CMV reactivation. Other surrogate markers may better predict functional recovery after treatment. Cytokines are directly affected by virus cytotoxicity and are used in the treatment of HIV. An understanding of the effects of antiviral therapy on cytokine expression is important(421) and may lead to more accurate monitoring of clinical disease. Published studies aiming to provide information on the changes in cytokine production with treatment are very variable and limited by small numbers and heterogeneous patient groups.

4.2 AIMS

This study aimed to document changes in cytokine profile of T cells in patients on combination ART. Screening for CMV retinitis in AIDS patients with low CD4 counts is widespread. The risk of CMV retinitis has, however, been observed to drop dramatically with the advent of combination ART, even in patients with only modest increases in their CD4 count. This study aimed to elucidate changes in immune function brought about by treatment with combination ART.

4.3 METHODS

4.3.1 Patient Selection

This was a cross sectional study in an urban general hospital and patients with HIV/AIDS were prospectively and sequentially recruited from the HIV outpatient clinic at Ealing Hospital from April 1999 to June 2000. The study was open to all patients attending the unit but patients who had received IL-2 therapy or cytotoxic chemotherapy were excluded. The Local Research Ethics Committee approved the study and written informed consent was obtained from all study participants. Blood samples were taken for analysis by flow cytometry at standard outpatient clinic visits.

4.3.2 Data Collected

The information collected on each patient included: gender, ethnicity, CDC classification(426), estimated duration of HIV infection, history of opportunistic infections, medication history and length of time on HAART, current CD4+ count and CD4+ nadir (defined as the lowest recorded CD4+ count), CD8+ count and current HIV viral load. For the purposes of this study, HAART was defined as consisting of at least three different antiretroviral drugs. Patients stable on no therapy had had no antiretroviral therapy for at least 6 months or were not yet considered to require HAART (in line with the British HIV Association guidelines (427)). Patients on HAART for less than three months were also considered as they are unlikely to have undergone full immune reconstitution and are generally considered to remain at risk of opportunistic infections during this period (428).

Absolute numbers of CD4+ and CD8+ T cells were determined by flow cytometry using a FACScan cytometer (Beckton Dickinson). HIV viral load was quantified using an ultrasensitive polymerase chain reaction (PCR) kit (Roche Diagnostic Systems; limit

of detection 50 HIV copies/ml). Peripheral blood was collected into sodium heparin containing vacutainer tubes and analysed within 2 hours without refrigeration.

Patients were divided into various sub-groups for analysis (see Figure 4.1). Comparisons were made between cytokine expression in the patient groups and that in samples from healthy laboratory workers ("Normals"). A number of patients provided samples on multiple occasions.

Cell stimulation, phenotypic and cytokine staining and flow cytometry were performed as described in Section 2.2.7.

Figure 4.1: Groups for Analysis

ANALYSIS	GROUPS
A	1. Normals 2. HIV Patients
B	1. Normals 2. Stable on no HAART 3. HAART
C	1. Normals 2. Stable on no HAART 3. HAART $\geq$ 3 months 4. HAART < 3months
D	1. Normals 2. Stable on no HAART 3. CD4>200 on HAART 4. CD4$\leq$200 on HAART
E	1. Normals 2. Stable on no HAART 3. CD4 increased > 100 from nadir on HAART 4. CD4 increased $\leq$100 from nadir on HAART
F	1. Normals 2. Stable on no HAART 3. HIV viral load $\leq$ 50 on HAART 4. HIV viral load > 50 on HAART
G	1. Normals 2. Stable on no HAART 3. HIV viral load $\leq$500 on HAART 4. HIV viral load >500 on HAART
H	1. Female Patients 2. Male Patients
I	1. British born 2. Sub-Saharan African 3. Other origin

4.3.3 Statistical Analysis

Statistical calculations were performed using SPSS for Windows (version 9.0-12.0 SPSS Inc, IL, USA). Non-parametric Kruskal-Wallis testing was used with Mann-Whitney testing to locate differences. In order to limit the number of comparisons Mann-Whitney testing was only used if a difference was seen with the Kruskal-Wallis test. Results are presented in Tables 3A-I. "N/A" denotes not applied and "Sig." denotes significance. $P<0.05$ was taken to be significant. A Bonferroni correction was used for multiple pairwise comparisons. Boxplots show the median, interquartile range, outliers and extreme cases.

In those patients providing repeat samples cytokine production at the first and second visit was compared using the Wilcoxon test for 2 related samples.

4.4 RESULTS

73 patients (49 male, 24 female) were analysed. 34 were men having sex with men; 37 were heterosexual; 1 was an intravenous drug user and 1 was a haemophiliac. Patients were at different stages of HIV infection and AIDS as shown in the Patient Characteristics tables. Antiretroviral therapy was prescribed in line with the British HIV Association (BHIVA) guidelines(427). All those on treatment were taking antiretroviral therapy involving 3 or more drugs and most patients were on treatment regimes involving at least one protease inhibitor or non-nucleoside reverse transcriptase inhibitor. This was designated as highly active anti-retroviral therapy (HAART). Regimens involving only nucleoside reverse transcriptase inhibitors all included abacavir.

Results are expressed as percentage production of cytokine by cell type eg: %CD4IL2= CD4+IL2+/[(CD4+IL2+) + (CD4+IL2-)] x 100. They were compared to those obtained from 15 healthy laboratory workers ("Normals" Group1; ages 22-47, 7 males, 8 females) and between patient sub-groups. Results are limited to the type 1 cytokines IL-2 and IFNγ as neither IL-4 or IL-10 expression were detected in patients and Normals.

4.4.1 ANALYSIS A: Effect of HIV Infection

HIV patients had significantly different IL-2 and IFNγ production from Normals. IL-2 production was markedly reduced in both CD4+ and CD8+ subsets (p=0.000 and p=0.001 respectively) in HIV patients. IFNγ production was increased in the CD4+ and CD8+ subsets (p=0.04 and p=0.02 respectively) in HIV patients. See Figure 4.2-4.7.

Figure 4.2: Patient Characteristics

Group	n	CDC Classification	Median Age (range) years	Median CD4 (range) Cells/mm^3	Median CD8 (range) Cells/mm^3	Median HIV Viral Load (range) RNA copies/ml
2. HIV Patients	73	A1x7; A2x6; A3x7; B1; B3x11; C2x3; C3x24; 14x?	39 (23-71)	250 (4-900)	860 (150-2570)	2270 (0-1960000)

Figure 4.3: Statistical Analysis; Normals vs HIV Patients

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Mann-Whitney Testing					
Normals vs HIV patients	Sig.	0.000	0.001	0.04	0.02

Figure 4.4: CD4 Cells Producing IL-2; Patients vs Normals

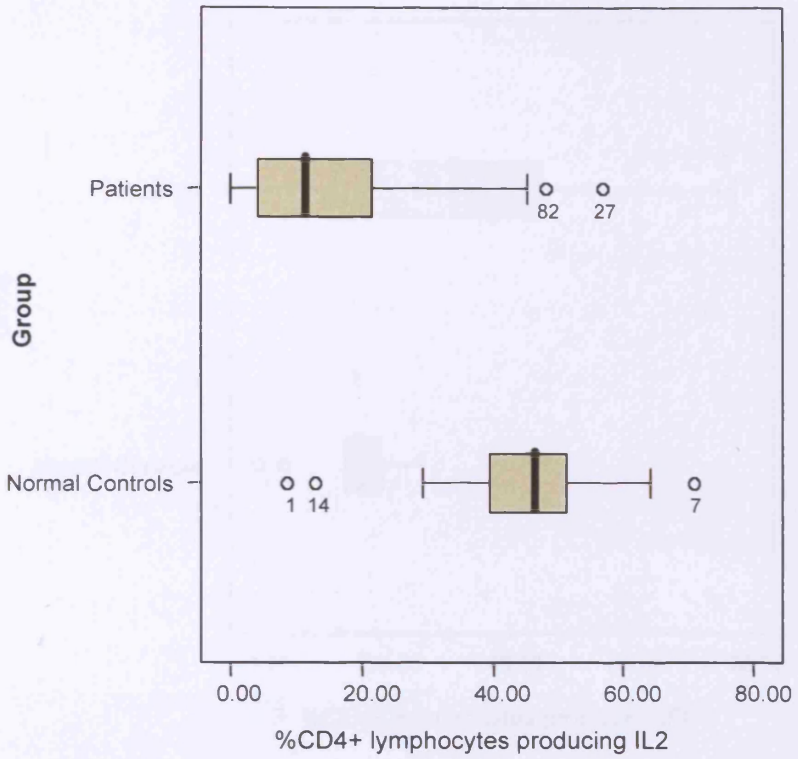


Figure 4.5: CD8 Cells Producing IL-2; Patients vs Normals

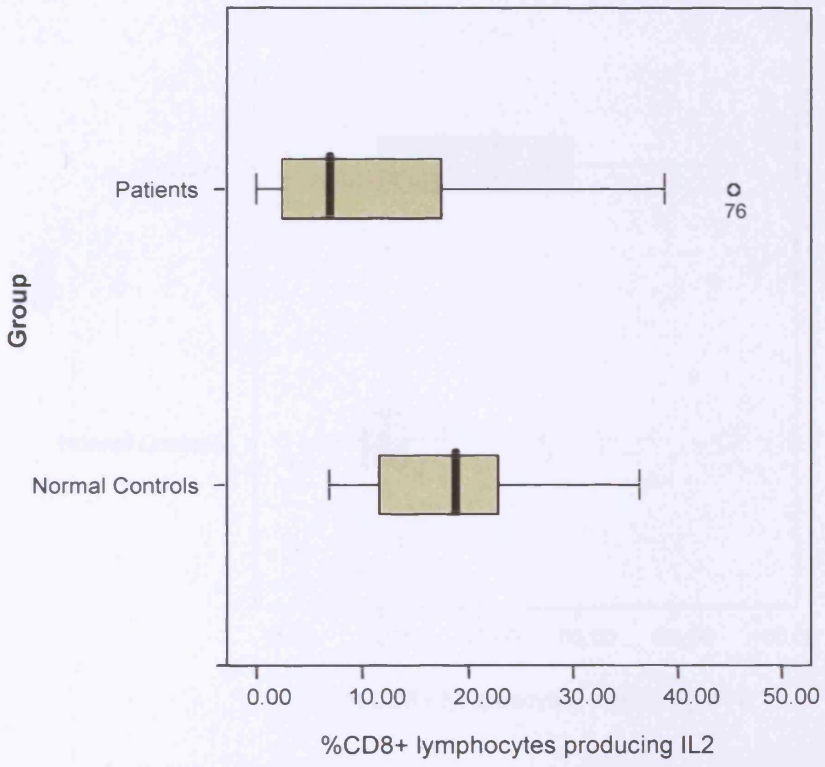


Figure 4.6: CD4 Cells Producing IFNγ; Pts vs Normals

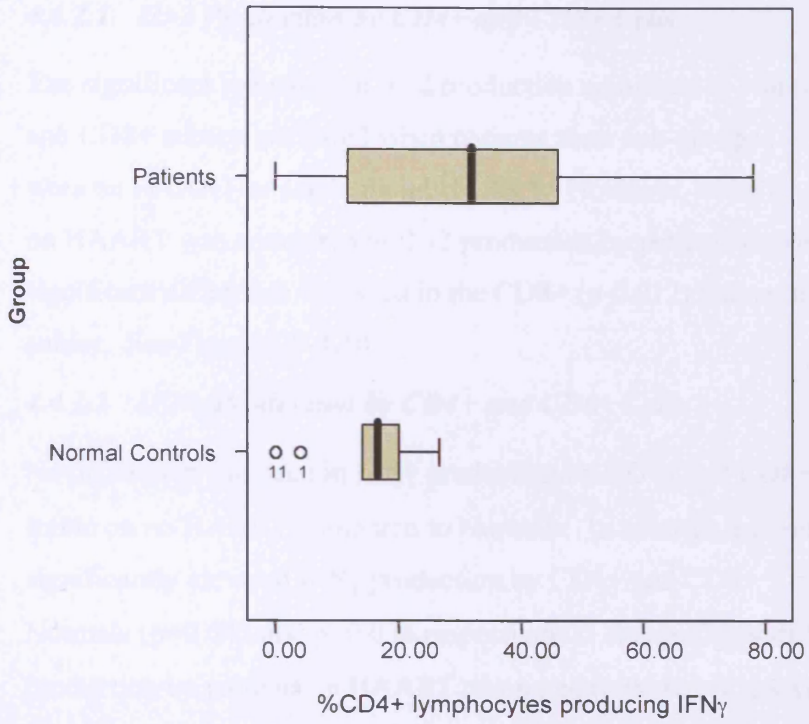
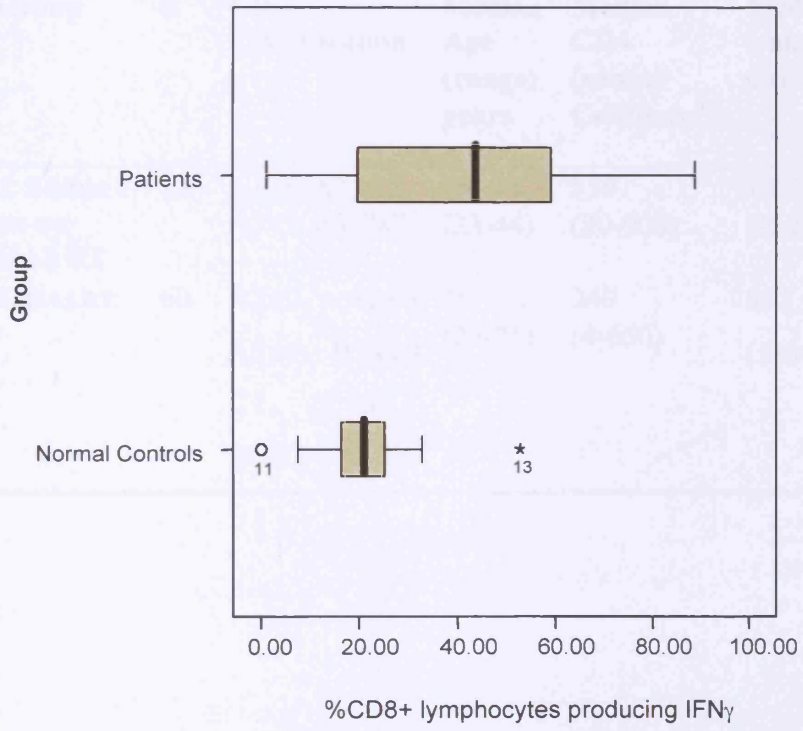


Figure 4.7: CD8 Cells Producing IFNγ; Pts vs Normals



4.4.2 ANALYSIS B: Effect of HAART

4.4.2.1 IL-2 Production by CD4+ and CD8+ Cells

The significant reduction in IL-2 production compared to Normals seen in both CD4+ and CD8+ subsets persisted when patients were sub-grouped according to whether they were on HAART or stable on no HAART. However, when IL-2 production by patients on HAART was compared to IL-2 production by patients stable on no HAART a significant difference was seen in the CD8+ (p-0.012) but not the CD4+ (p=0.459) subset. See Figure 4.8-4.10.

4.4.2.2 IFNγ Production by CD4+ and CD8+ Cells

No difference was seen in IFNγ production by CD4+ and CD8+ cells of HIV patients stable on no HAART compared to Normals. In contrast, patients on HAART had significantly elevated IFNγ production by CD4+ and CD8+ T cells compared to Normals (p=0.045 and p=0.036 respectively). A significant difference in IFNγ production by patients on HAART compared to those off HAART was not seen. See Figure 4.9.

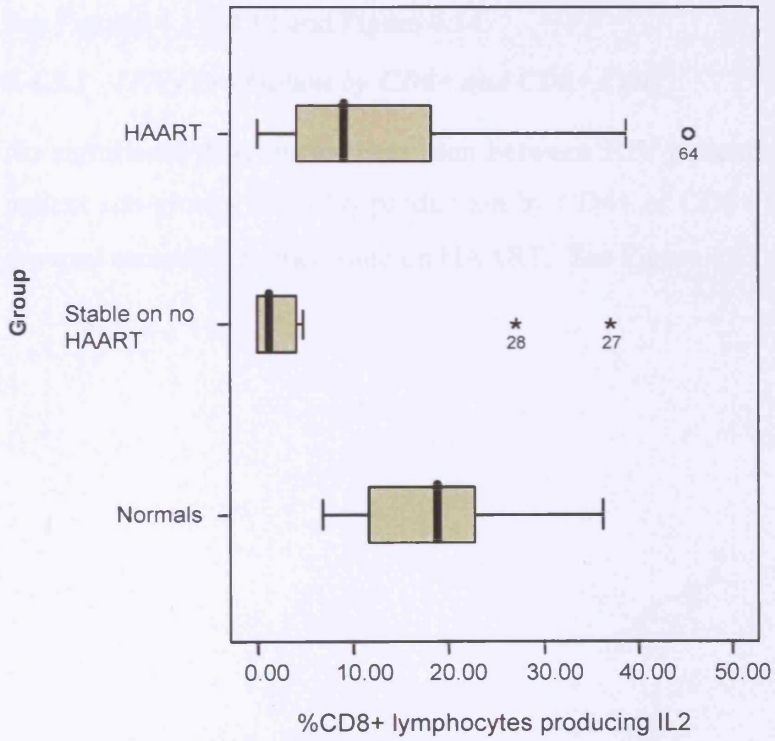
Figure 4.8: Patient Characteristics; HAART vs No HAART

Group	n	CDC Classification	Median Age (range) years	Median CD4 (range) Cells/mm^3	Median CD8 (range) Cells/mm^3	Median HIV Viral Load (range) copies/ml
2. Stable on no HAART	13	A1x5, A2, C2, A3x3, B3, ?x2	35 (23-44)	530 (80-900)	1000 (450-2570)	43600 (1 260-1 000 000)
3. HAART	60	A1x2, A2x5, A3x4, B3x10, C2, C3x25, ?x13	39 (23-71)	240 (4-650)	800 (150-2280)	973 (50-1 960 000)

Figure 4.9: Statistical Analysis; Effect of HAART

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-3)	χ^2 df Sig.	25.842 2 0.000	16.593 2 0.000	6.528 2 0.038	5.987 2 0.050
Mann-Whitney Testing					
1 vs 2	Sig. (Corrected)	0.000 (0.000)	0.001 (0.003)	0.828 (1)	0.321 (0.963)
1 vs 3	Sig. (Corrected)	0.000 (0.000)	0.005 (0.015)	0.015 (0.045)	0.012 (0.036)
2 vs 3	Sig. (Corrected)	0.153 (0.459)	0.004 (0.012)	0.163 (0.489)	0.485 (1)

Figure 4.10: CD8 Cells Producing IL-2; Effect of HAART



4.4.3 ANALYSIS C: Time on HAART

4.4.3.1 IL-2 Production by CD4+ Cells

HIV patients produced very significantly reduced IL-2 levels compared to Normals. Further analysis revealed no significant differences between patient sub-groups irrespective of time on HAART. See Figure 4.11–4.13.

4.4.3.2 IL-2 Production by CD8+ Cells

Patients stable on no HAART were found to have significantly reduced IL-2 production by CD8+ cells compared to Normals ($p=0.006$). Patients on HAART for less than 3 months were also seen to have significantly reduced IL-2 production by CD8+ cells compared to Normals ($p=0.012$). This difference was not seen when comparing patients on HAART for at least 3 months and Normals.

Patients stable on no HAART were seen to have significantly less IL-2 production by CD8+ cells from patients who had been on HAART for at least 3 months ($p=0.024$). See Figures 4.11, 4.12 and Figure 4.14.

4.4.3.3 IFNγ Production by CD4+ and CD8+ Cells

No significant differences were seen between HIV patients and Normals or between patient sub-groups for IFNγ production by CD4+ or CD8+ cells when patients where grouped according to their time on HAART. See Figure 4.12.

Figure 4.11: Patient Characteristics; Time on HAART

Group	n	CDC Classification	Median Age (range) years	Median CD4 (range) cells/mm3	Median CD8 (range) cells/mm3	Median HIV Viral Load (range) RNA copies/ml
2. Stable on no HAART	13	A1x5, A2, C2, A3x3, B3, ?x2	35 (23-44)	530 (80-900)	1000 (450-2570)	43 600 (1 260-1 000 000)
3. HAART ≥ 3 months	54	A1x2, A2x5, A3x4, B3x9, C2, C3x20, ?x13	39 (23-71)	250 (4-650)	860 (150-2280)	659 (0-692 460)
4. HAART < 3 months	6	C3x5, B3	42 (31-57)	35 (9-120)	305 (260-630)	22 400 (1 390-1 960 000)

Figure 4.12: Statistical Analysis; Time on HAART

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-4)	χ^2 df Sig.	27.760 3 0.000	18.649 3 0.000	7.533 3 0.057	6.023 3 0.111
Comparing patient groups (2-4)	χ^2 df Sig.	4.541 2 0.103	9.944 2 0.007	N/A - -	N/A - -
Mann-Whitney Testing					
1 vs 2	Sig. (Corrected)	0.000 (0.000)	0.001 (0.006)	N/A	N/A
1 vs 3	Sig. (Corrected)	0.000 (0.000)	0.012 (0.072)	N/A	N/A
1 vs 4	Sig. (Corrected)	0.001 (0.003)	0.002 (0.012)	N/A	N/A
2 vs 3	Sig. (Corrected)	N/A	0.004 (0.024)	N/A	N/A
2 vs 4	Sig. (Corrected)	N/A	0.157 (0.942)	N/A	N/A
3 vs 4	Sig. (Corrected)	N/A	0.117 (0.702)	N/A	N/A

Figure 4.13: CD4 Cells Producing IL2; Effect of Time on HAART

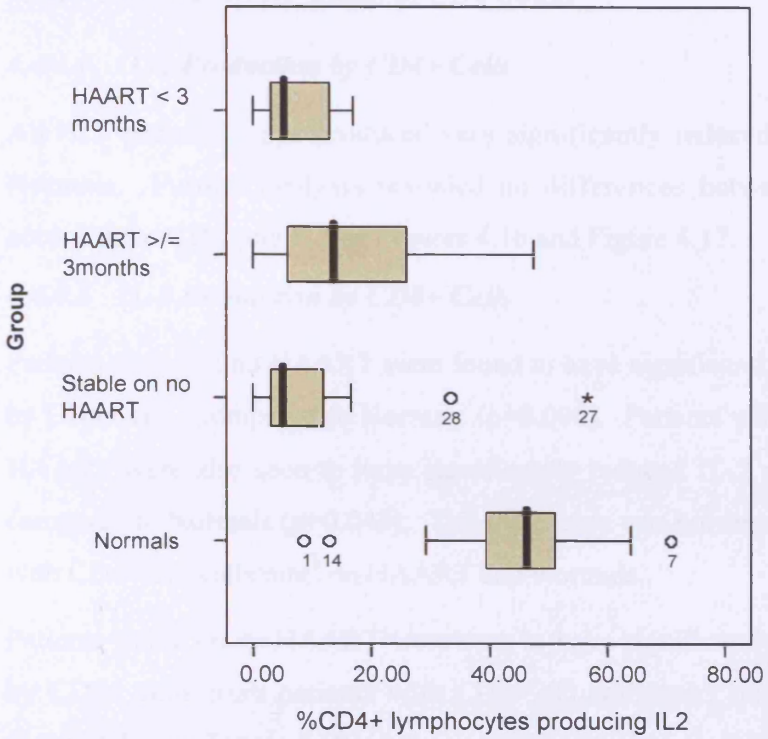
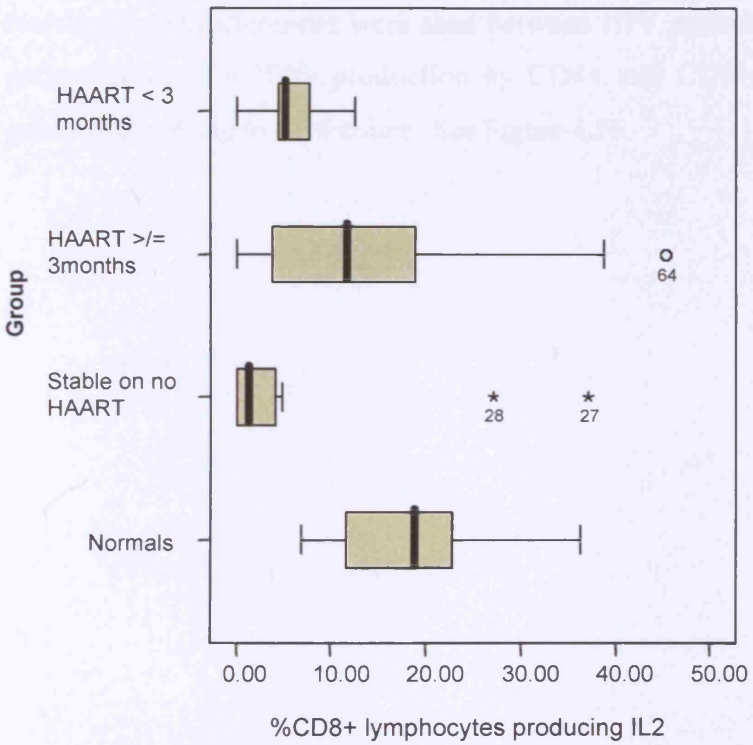


Figure 4.14: CD8 Cells Producing IL2; Effect of Time on HAART



4.4.4 ANALYSIS D: Effect of CD4 Count

4.4.4.1 IL-2 Production by CD4+ Cells

All HIV patient groups produced very significantly reduced IL-2 levels compared to Normals. Further analysis revealed no differences between patients sub-grouped according to CD4 count. See Figures 4.16 and Figure 4.17.

4.4.4.2 IL-2 Production by CD8+ Cells

Patients stable on no HAART were found to have significantly reduced IL-2 production by CD8+ cells compared to Normals ($p=0.006$). Patients with CD4≤200 cells/mm^3 on HAART were also seen to have significantly reduced IL-2 production by CD8+ cells compared to Normals ($p=0.048$). This difference was not seen when comparing patients with CD4>200 cells/mm^3 on HAART and Normals.

Patients stable on no HAART were seen to have significantly reduced IL-2 production by CD8+ cells from patients with CD4>200 cells/mm3 on HAART ($p=0.030$). See Figure 4.16 and Figure 4.18.

4.4.4.3 IFNγ Production by CD4+ and CD8+ Cells

No significant differences were seen between HIV patients and Normals or between patient groups for IFNγ production by CD4+ and CD8+ cells when patients were grouped according to CD4 count. See Figure 4.16.

Figure 4.15: Patient Characteristics; CD4 Count

Group	n	CDC Classification	Median Age (range) years	Median CD4 (range) cells/mm^3	Median CD8 (range) cells/mm^3	Median HIV Viral Load (range) copies/ml
2. Stable on no HAART	13	A1x5, A2, C2, A3x3, B3, ?x2	35 (23-44)	530 (80-900)	1000 (450-2570)	43 600 (1 260-1 000 000)
3. CD4 > 200 on HAART	35	A1x2, A2x5, A3x3, B3x4, C2, C3x11, ?x9	38.5 (23-71)	360 (210-650)	910 (350-2280)	616 (0-133 879)
4. CD4 ≤ 200 on HAART	25	A3, B3x7, C3x13, ?x4	39.5 (26-68)	90 (4-200)	550 (150-2120)	2750 (0-1 960 000)

Figure 4.16: Statistical Analysis; CD4 Count

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-4)	**χ^2**	28.298	17.187	6.993	6.444
	df	3	3	3	3
	Sig.	0.000	0.001	0.072	0.092
Comparing patient groups (2-4)	**χ^2**	5.393	8.810	N/A	N/A
	df	2	2	-	-
	Sig.	0.067	0.012	-	-
Mann-Whitney Testing					
1 vs 2	**Sig. (corrected)**	0.000 (0.000)	0.001 (0.006)	N/A	N/A
1 vs 3	**Sig. (corrected)**	0.000 (0.000)	0.013 (0.078)	N/A	N/A
1 vs 4	**Sig. (corrected)**	0.000 (0.000)	0.008 (0.048)	N/A	N/A
2 vs 3	**Sig. (corrected)**	N/A	0.005 (0.030)	N/A	N/A
2 vs 4	**Sig. (corrected)**	N/A	0.020 (0.120)	N/A	N/A
3 vs 4	**Sig. (corrected)**	N/A	0.349 (1)	N/A	N/A

Figure 4.17: CD4 Cells Producing IL2; Effect of CD4 Count

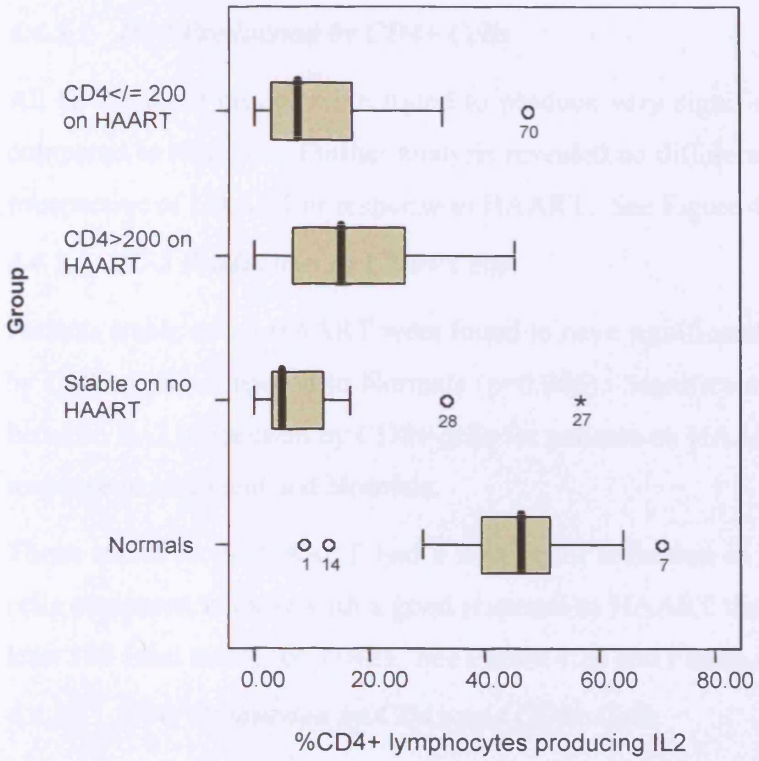
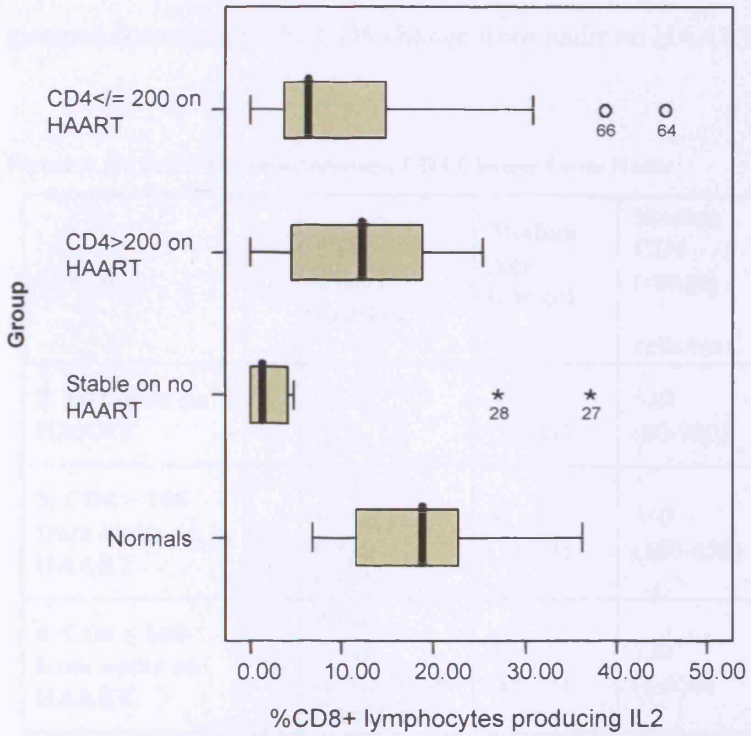


Figure 4.18: CD8 Cells Producing IL2; Effect of CD4 Count



4.4.5 ANALYSIS E: CD4 Change from Nadir

4.4.5.1 IL-2 Production by CD4+ Cells

All HIV patient groups were found to produce very significantly reduced IL-2 levels compared to Normals. Further analysis revealed no differences between patient groups irrespective of HAART or response to HAART. See Figure 4.20 and Figure 4.21.

4.4.5.2 IL-2 Production by CD8+ Cells

Patients stable on no HAART were found to have significantly reduced IL-2 production by CD8+ cells compared to Normals (p=0.006). Significant differences were not seen between IL-2 production by CD8+ cells for patients on HAART irrespective of the CD4 response to treatment and Normals.

Those stable on no HAART had a significant reduction in IL-2 production by CD8+ cells compared to those with a good response to HAART therapy (CD4 increased by at least 100 from nadir) (p=0.042). See Figure 4.20 and Figure 4.22.

4.4.5.3 IFNγ Production by CD4+ and CD8+ Cells

No significant differences were seen between HIV patients and Normals or between patient sub-groups for IFNγ production by CD4+ and CD8+ cells when patients were grouped according to the CD4 change from nadir on HAART. See Figure 4.20.

Figure 4.19: Patient Characteristics; CD4 Change From Nadir

Group	n	CDC Classi-fication	Median Age (range) years	Median CD4 (range) cells/mm^3	Median CD8 (range) cells/mm^3	Median HIV Viral Load (range) RNA copies/ml
2. Stable on no HAART	13	A1x5, A2, C2, A3x3, B3, ?x2	35 (23-44)	530 (80-900)	1000 (450-2570)	43 600 (1 260-1 000 000)
3. CD4 > 100 from nadir on HAART	27	A2x3, A3x2, B3x5, C2x2, C3x11, ?x5	41 (23-53)	360 (180-650)	990 (320-2280)	400 (0-95 200)
4. CD4 ≤ 100 from nadir on HAART	30	A1x2, A2x2, A3x2, B3x6, C3x12, ?x6	39 (26-71)	120 (4-650)	620 (150-2120)	3190 (0-1 960 000)

Figure 4.20: Statistical Analysis; CD4 Change from Nadir

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-4)	χ^2 df Sig.	25.805 3 0.000	15.812 3 0.001	5.99 3 0.112	6.378 3 0.095
Comparing patient groups (2-4)	χ^2 df Sig.	2.032 2 0.362	7.750 2 0.021	N/A - -	N/A - -
Mann-Whitney Testing					
1 vs 2	Sig. (corrected)	0.000 (0.000)	0.001 (0.006)	N/A	N/A
1 vs 3	Sig. (corrected)	0.000 (0.000)	0.016 (0.096)	N/A	N/A
1 vs 4	Sig. (corrected)	0.000 (0.000)	0.015 (0.090)	N/A	N/A
2 vs 3	Sig. (corrected)	N/A	0.007 (0.042)	N/A	N/A
2 vs 4	Sig. (corrected)	N/A	0.017 (0.102)	N/A	N/A
3 vs 4	Sig. (corrected)	N/A	0.838 (1)	N/A	N/A

Figure 4.21: CD4 Cells Producing IL2; Effect of CD4 Change from Nadir

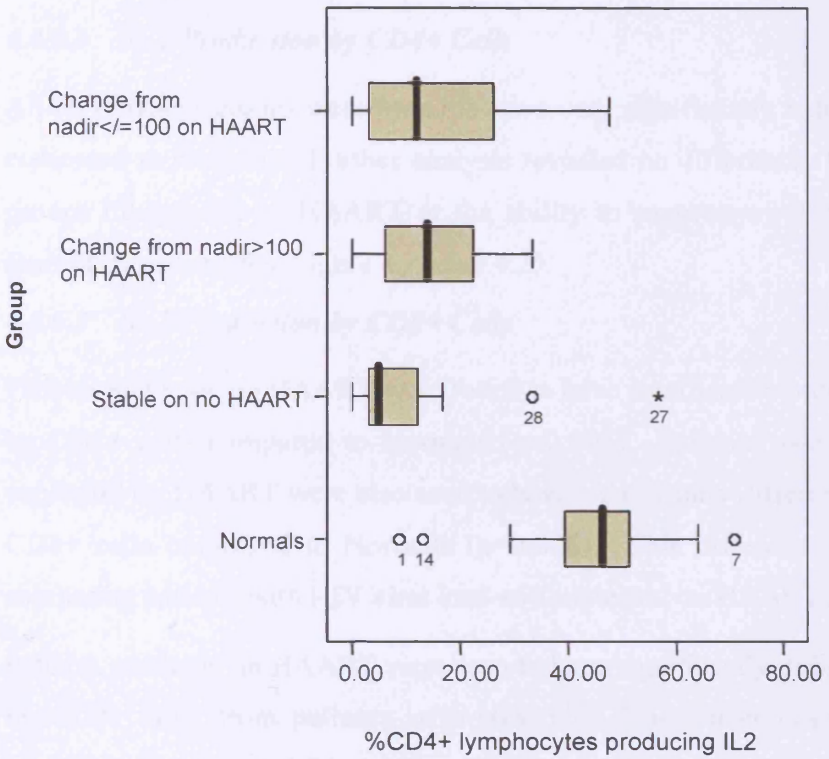
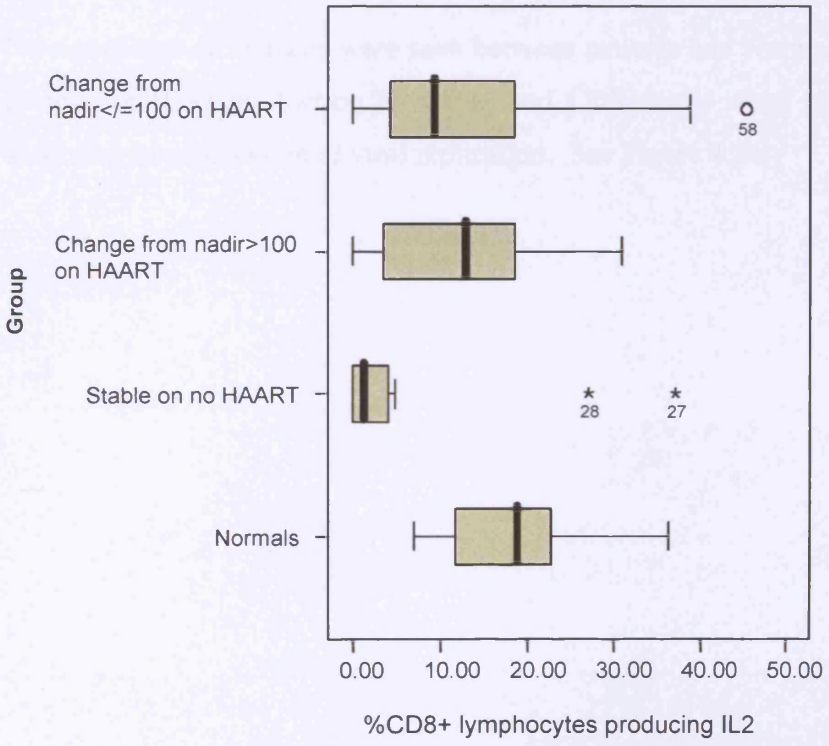


Figure 4.22: CD8 Cells Producing IL2; Effect of CD4 Change from Nadir



4.4.6 ANALYSIS F: HIV Viral Load 50 copies/ml

4.4.6.1 IL-2 Production by CD4+ Cells

All HIV patient groups were found to have very significantly reduced IL-2 production compared to Normals. Further analysis revealed no differences between patient sub-groups irrespective of HAART or the ability to suppress viral replication below the limit of detection. See Figure 4.24 and 4.25.

4.4.6.2 IL-2 Production by CD8+ Cells

Patients stable on no HAART were found to have significantly reduced IL-2 production by CD8+ cells compared to Normals ($p=0.006$). Patients with HIV viral load >50 copies/ml on HAART were also seen to have significantly different IL-2 production by CD8+ cells compared to Normals ($p=0.018$). This difference was not seen when comparing patients with HIV viral load ≤50 copies/ml on HAART and Normals ($p=1$).

Patients stable on no HAART were seen to have significantly different IL-2 production by CD8+ cells from patients with HIV viral load>50 copies/ml despite HAART ($p=0.030$). See Figure 4.24 and Figure 4.26.

4.4.6.3 IFNγ Production by CD4+ and CD8+ Cells

No significant differences were seen between patients and Normals or between patient groups for IFNγ production by CD4+ and CD8+ cells when patients were grouped according to suppression of viral replication. See Figure 4.24.

Figure 4.23: Patient Characteristics; HIV Viral load (50)

Group	n	CDC Classi-fication	Median Age (range) years	Median CD4 (range) cells/mm^3	Median CD8 (range) cells/mm^3	Median HIV Viral Load (range) RNA copies/ml
2. Stable on no HAART	13	A1x5, A2, C2, A3x3, B3, ?x2	35 (23-44)	530 (80-900)	1000 (450-2570)	43 600 (1 260-1 000 000)
3. HIV viral load ≤ 50 on HAART	12	A1, A2, A3, B3x2, C3x5, ?x2	26 (23-53)	290 (30-650)	49 (320-1910)	3 (0-50)
4. HIV viral load > 50 on HAART	48	A1, A2x4, A3x4, B3x8, C2, C3x18, ?x12	39 (29-71)	230 (4-650)	825 (150-2280)	2500 (92-1 960 000)

Figure 4.24: Statistical Analysis; HIV Viral Load (50)

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-4)	χ^2 df Sig.	25.379 3 0.000	16.875 3 0.001	6.835 3 0.077	6.073 3 0.108
Comparing patient groups (2-4)	χ^2 df Sig.	2.638 2 0.267	8.441 2 0.015	N/A - -	N/A - -
Mann-Whitney Testing					
1 vs 2	Sig. (corrected)	0.000 (0.000)	0.001 (0.006)	N/A	N/A
1 vs 3	Sig. (corrected)	0.001 (0.003)	0.205 (1)	N/A	N/A
1 vs 4	Sig. (corrected)	0.000 (0.000)	0.003 (0.018)	N/A	N/A
2 vs 3	Sig. (corrected)	N/A	0.025 (0.150)	N/A	N/A
2 vs 4	Sig. (corrected)	N/A	0.005 (0.030)	N/A	N/A
3 vs 4	Sig. (corrected)	N/A	0.572 (1)	N/A	N/A

Figure 4.25: CD4 Cells Producing IL2; Effect of HIV Viral Load (50)

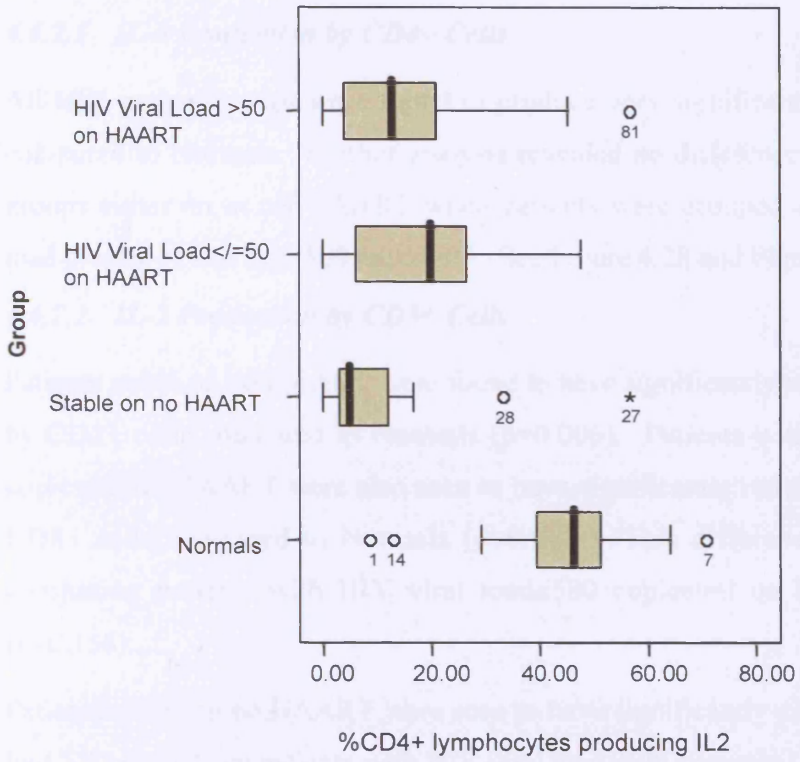
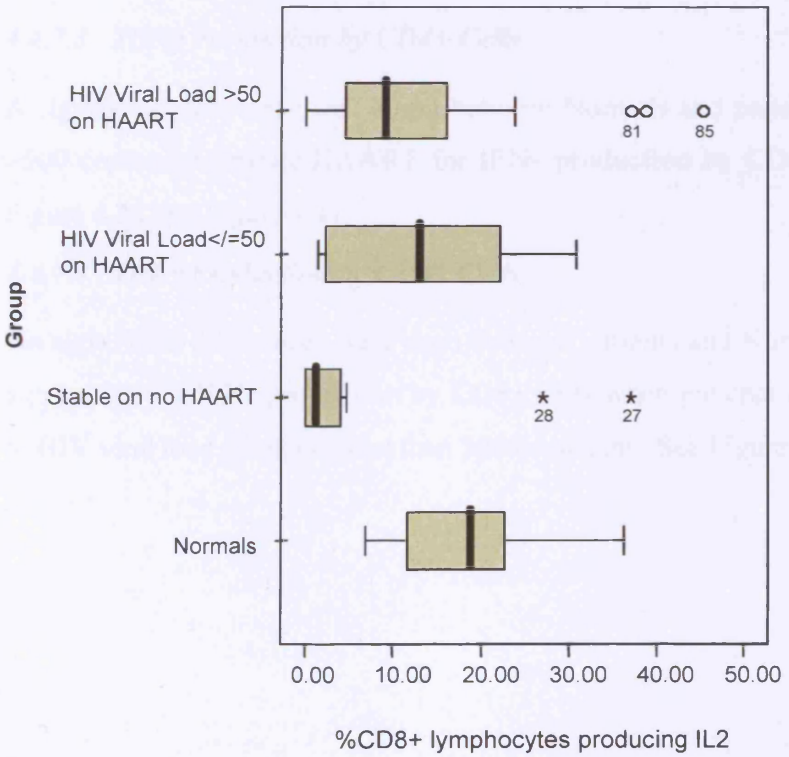


Figure 4.26: CD8 Cells Producing IL2; Effect of HIV Viral Load (50)



4.4.7 ANALYSIS G: HIV Viral Load 500 copies/ml

4.4.7.1 IL-2 Production by CD4+ Cells

All HIV patient groups were found to produce very significantly reduced IL-2 levels compared to Normals. Further analysis revealed no differences between patient sub-groups either on or off HAART when patients were grouped according to HIV viral load greater or less than 500 copies/ml. See Figure 4.28 and Figure 4.29.

4.4.7.2 IL-2 Production by CD8+ Cells

Patients stable on no HAART were found to have significantly reduced IL-2 production by CD8+ cells compared to Normals (p=0.006). Patients with HIV viral load >500 copies/ml on HAART were also seen to have significantly reduced IL-2 production by CD8+ cells compared to Normals (p=0.042). This difference was not seen when comparing patients with HIV viral load≤500 copies/ml on HAART and Normals (p=0.156).

Patients stable on no HAART were seen to have significantly different IL-2 production by CD8+ cells from patients with HIV viral load>500 copies/ml on HAART (p=0.018). See Figure 4.28 and Figure 4.30.

4.4.7.3 IFNγ Production by CD4+ Cells

A significant difference was found between Normals and patients with HIV viral load >500 copies/ml despite HAART for IFNγ production by CD4 cells (p=0.012). See Figure 4.28 and Figure 4.31.

4.4.7.4 IFNγ Production by CD8+ Cells

No significant differences were seen between patients and Normals or between patient sub-groups for IFNγ production by CD8+ cells when patients were grouped according to HIV viral load greater or less than 500 copies/ml. See Figure 4.28.

Figure 4.27: Patient Characteristics; HIV Viral load (500)

Group	n	CDC Classification	Median Age (range) years	Median CD4 (range) cells/mm^3	Median CD8 (range) cells/mm^3	Median HIV Viral Load (range) RNA copies/ml
2. Stable on no HAART	13	A1x5, A2, C2, A3x3, B3, ?x2	35 (23-44)	530 (80-900)	1000 (450-2570)	43 600 (1 260-1 000 000)
3. HIV viral load ≤ 500 on HAART	24	A1x2, A2x3, A3x3, B3x3, C3x9, ?x4	39 (23-71)	310 (20-650)	860 (320-2120)	71 (0-434)
4. HIV viral load >500 on HAART	36	A2x2, A3x2, B3x7, C2x1, C3x14, ?x10	39 (29-68)	210 (4-640)	760 (150-2280)	4051 (560-1 960 000)

Figure 4.28: Statistical Analysis; HIV Viral Load (500)

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-4)	χ^2 df sig.	24.973 3 0.000	16.524 3 0.001	10.200 3 0.017	7.358 3 0.061
Comparing patient groups (2-4)	χ^2 df sig.	2.147 2 0.342	8.254 2 0.016	5.382 2 0.068	N/A
Mann-Whitney Testing					
1 vs 2	Sig. (corrected)	0.000 (0.000)	0.001 (0.006)	0.828 (1)	N/A
1 vs 3	Sig. (corrected)	0.000 (0.000)	0.026 (0.156)	0.212 (0.636)	N/A
1 vs 4	Sig. (corrected)	0.000 (0.000)	0.007 (0.042)	0.002 (0.012)	N/A
2 vs 3	Sig. (corrected)	N/A	0.035 (0.210)	N/A	N/A
2 vs 4	Sig. (corrected)	N/A	0.003 (0.018)	N/A	N/A
3 vs 4	Sig. (corrected)	N/A	0.957 (1)	N/A	N/A

Figure 4.29: CD4 Cells Producing IL2; Effect of HIV Viral Load (500)

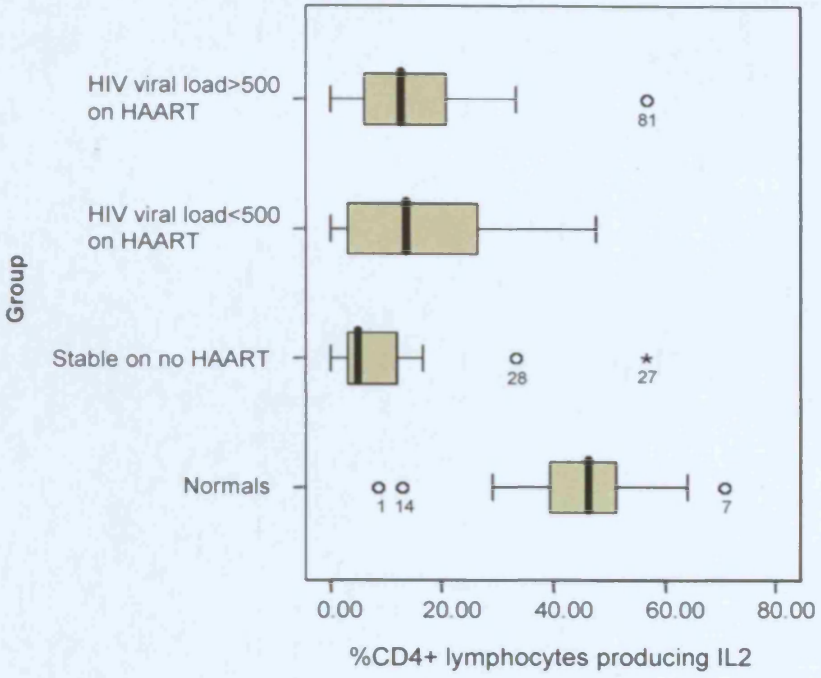


Figure 4.30: CD8 Cells Producing IL2; Effect of HIV Viral Load (500)

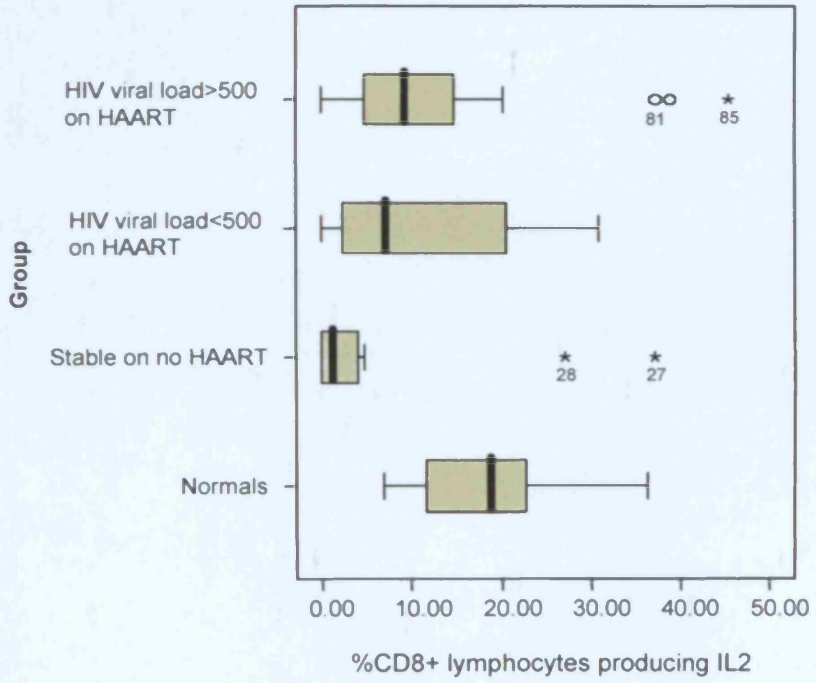
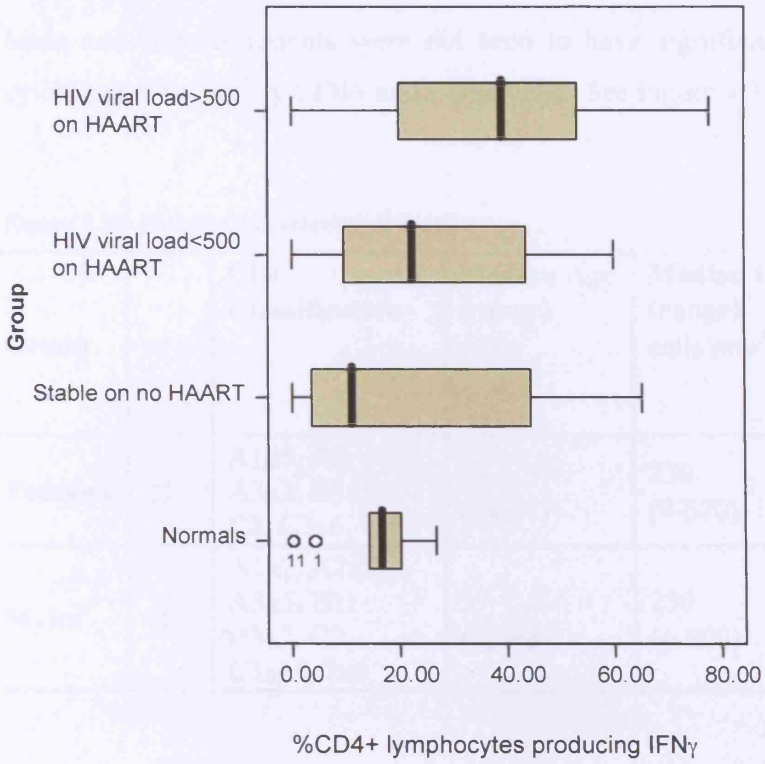


Figure 4.31: CD4 Cells Producing IFNγ; Effect of HIV Viral Load (500)



4.4.8 ANALYSIS H: Gender

Male and female patients were not seen to have significant differences in type 1 cytokine production by CD4+ and CD8+ cells. See Figure 4.32.

Figure 4.32: Patient Characteristics; Gender

Group	**n**	**CDC Classification**	**Median Age (range)** years	**Median CD4 (range)** cells/mm^3	**Median HIV Viral Load (range)** RNA copies/ml
Females	25	A1x5, A2, A3x2, B3x5, C2, C3x6, ?x5	36 (24-71)	230 (9-670)	904 (0-220 036)
Males	48	A1x2, A2x5, A3x5, B1, B3x7, C2, C3x18, ?x9	39 (23-68)	250 (4-900)	2565 (0-1 960 000)

Figure 4.33: Statistical Analysis; Gender

		CD4IL2	**CD8IL2**	**CD4IFN**	**CD8IFN**
Mann-Whitney Testing					
Comparing Female to Male Pts	Sig.	0.373	0.236	0.641	0.905

4.4.9 ANALYSIS I: Origin

Analysis of patients' origin (British born, Sub-Saharan African or Other) revealed no significant differences in type 1 cytokine production by CD4+ or CD8+ cells in the different groups. See Figure 4.35.

Figure 4.34: Patient Characteristics; Origin

Group	n	CDC Classi-fication	Median Age (range) years	Median CD4 (range) cells/mm3	Median HIV Viral Load (range) RNA copies/ml
1.British Born	34	A1x4, A2x3, A3x4, B3x4, C2x1, C3x11, ?x7	42 (23-71)	325 (10-900)	2230 (0-692 460)
2. Sub-Saharan African	24	A1, A2, A3x2, B3x7, C2x1, C3x8, ?x4	38 (24-54)	165 (20-670)	3190 (0-1 960 000)
3. Other Origin	15	A1x2, A2x2, A3, B1, B3, C3x5, ?x3	33 (23-57)	250 (4-880)	1260 (0-192 000)

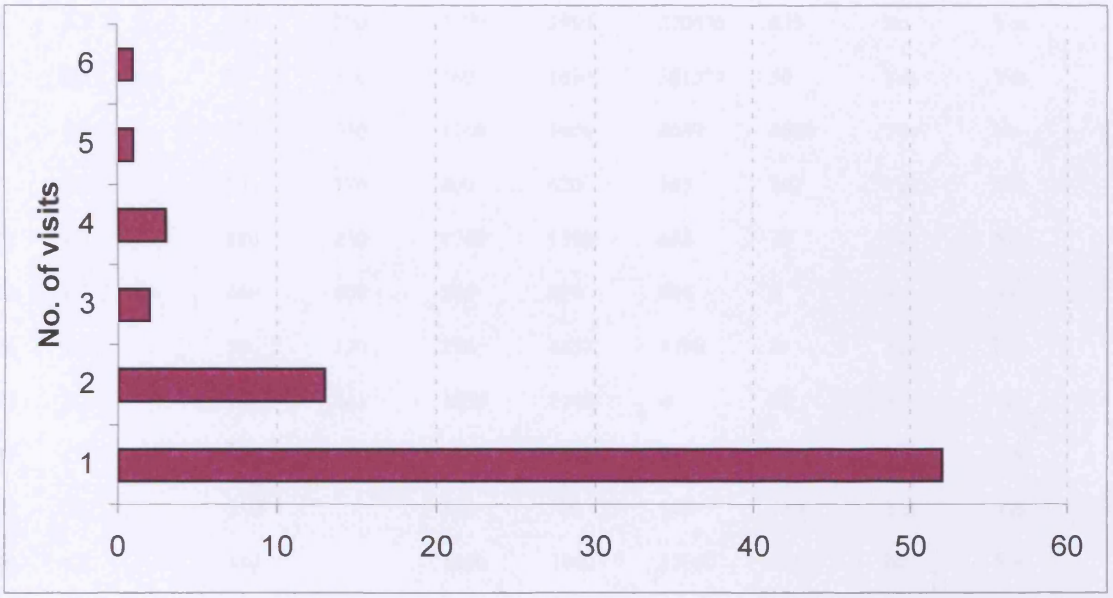
Figure 4.35: Statistical Analysis; Origin

		CD4IL2	CD8IL2	CD4IFN	CD8IFN
Kruskal-Wallis Testing					
Comparing all groups (1-3)	$\chi 2$ df Sig.	2.493 2 0.288	1.313 2 0.519	0.251 2 0.882	1.550 2 0.461
Mann-Whitney Testing					
1 vs 2		N/A	N/A	N/A	N/A
1 vs 3		N/A	N/A	N/A	N/A
2 vs 3		N/A	N/A	N/A	N/A

4.4.10 ANALYSIS J: Repeat Samples

14 patients were investigated for cytokine production on 2 occasions, 2 on 3 occasions, 3 on 4 occasions, 1 on 5 occasions and 1 on 6 occasions (See Figure 4.36).

Figure 4.36: Frequency of Attendance



4.4.10.1 2 Visits

14 patients were investigated for cytokine production on 2 occasions. One patient was excluded because the results of his first analysis were lost. Patient characteristics at each visit are documented in Figure 4.36. No patient changed CDC classification between visits. Patient 3 and patient 40 were on no antiretroviral therapy at the first visit, the other patients were on HAART regimes. All patients were on HAART at the second visit. The second visit was 119 days after the first for patient 3 and 315 days after the first for patient 40. The median interval between visits was 126 days (range 28-336 days).

No significant differences were seen in IL-2 or IFNγ production by CD4 or CD8 cells when comparing the first and second visit (Figures 4.38-4.42). Cytokine production was noted to be stable despite observed improvements in CD4 count, CD8 count and HIV viral load between visits (Figure 4.37)

Figure 4.37: 2 Visits; Patient Characteristics

Pt no.	CDC Classification	CD4 count (Visit 1)	CD4 count (Visit 2)	CD8 count (Visit 1)	CD8 count (Visit 2)	HIV Load (Visit 1)	HIV Load (Visit2)	HAART (Visit 1)	HAART (Visit 2)
3	A3	150	200	2570	2990	220036	635	No	Yes
4	C3	50	370	260	1030	361327	30	Yes	Yes
6	C3	170	250	1240	1600	4649	6600	Yes	No
7	B3	60	170	400	620	185	202	Yes	Yes
22	C3	180	250	1740	1390	668	25	Yes	Yes
23	C2	640	400	860	620	904	0	Yes	Yes
26	C3	10	120	270	1650	1390	0	Yes	Yes
27	B3	180	461	1050	1140	0	90	Yes	Yes
33	C3	300	270	1720	1390	0	0	Yes	Yes
38	?2	250		860	720	109	136	Yes	Yes
40	C2	440		1560	1040	57000	4250	No	Yes
46	C3	420		1050	970	153	9410	Yes	Yes
47	C3	330		740	n/a	0	2230	Yes	Yes
	Median	180	270	1050	1090	668	136		
	Minimum	10	120	260	620	0	0		
	Maximum	640	510	2570	2990	361327	9410		

Figure 4.38: CD4 Cells Producing IL2: First Visit compared to Second Visit

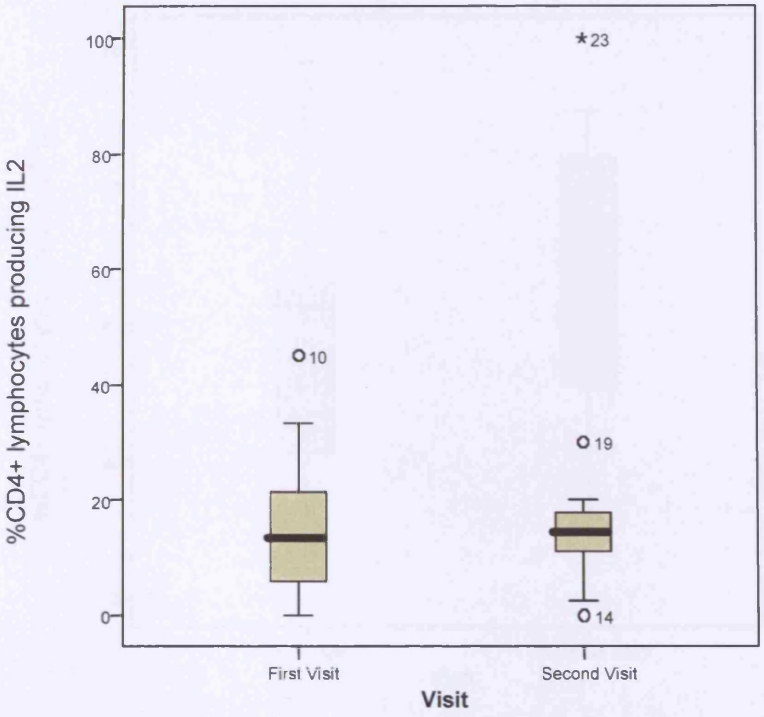


Figure 4.39: CD8 Cells Producing IL2: First Visit compared to Second Visit

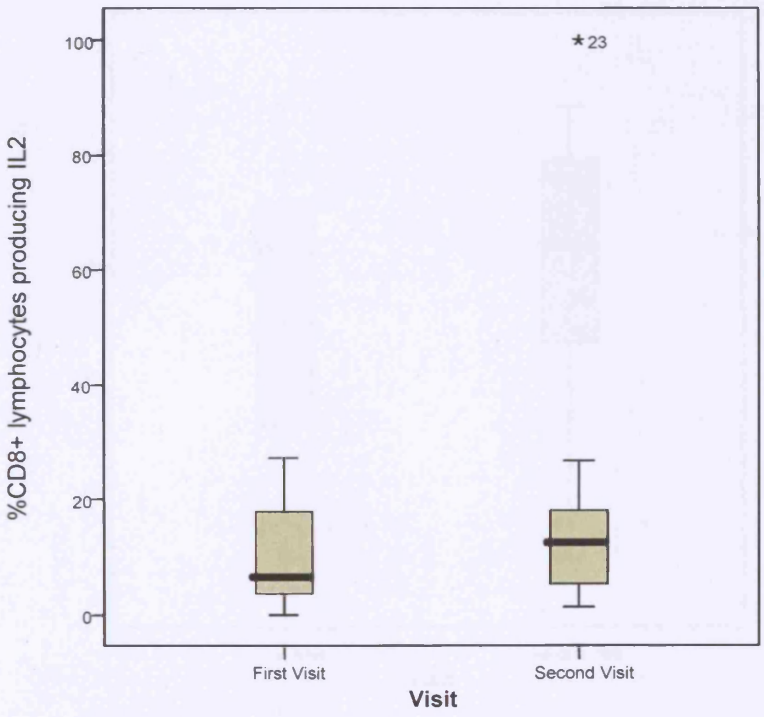


Figure 4.40: CD4 Cells Producing IFNγ: First Visit compared to Second Visit

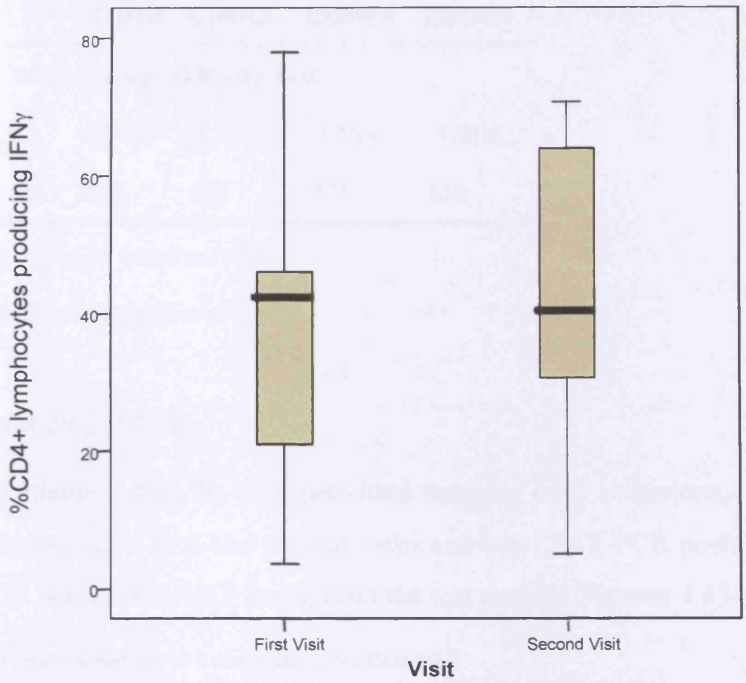


Figure 4.41: CD8 Cells Producing IFNγ: First Visit compared to Second Visit

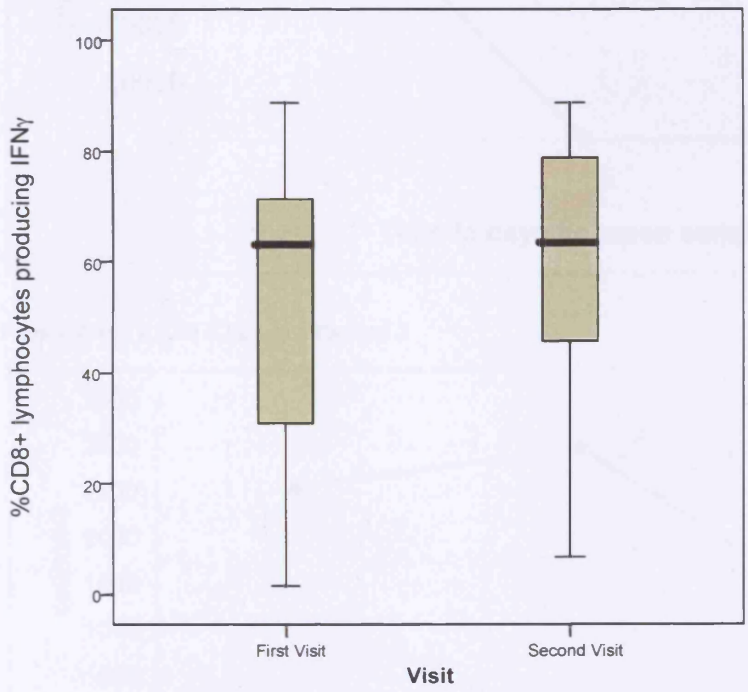


Figure 4.42: Statistical Analysis; First vs Second Visit

	CD4IL2	CD8IL2	CD4IFN	CD8IFN
Wilcoxon Signed Ranks Test				
Z	-.524(a)	-1.223(a)	-.622(a)	-.178(b)
Sig.	.600	.221	.534	.859

a Based on negative ranks.

b Based on positive ranks.

4.4.10.2 3 Visits

Patients 3 and 20 both provided samples on 3 occasions. Patient 3 started HAART between the first and second visits and was CMV PCR positive at the first visit. Patient 20 was on HAART throughout the test period. Figures 4.43-4.47.

Figure 4.43: Viral Load Changes; Patient 3

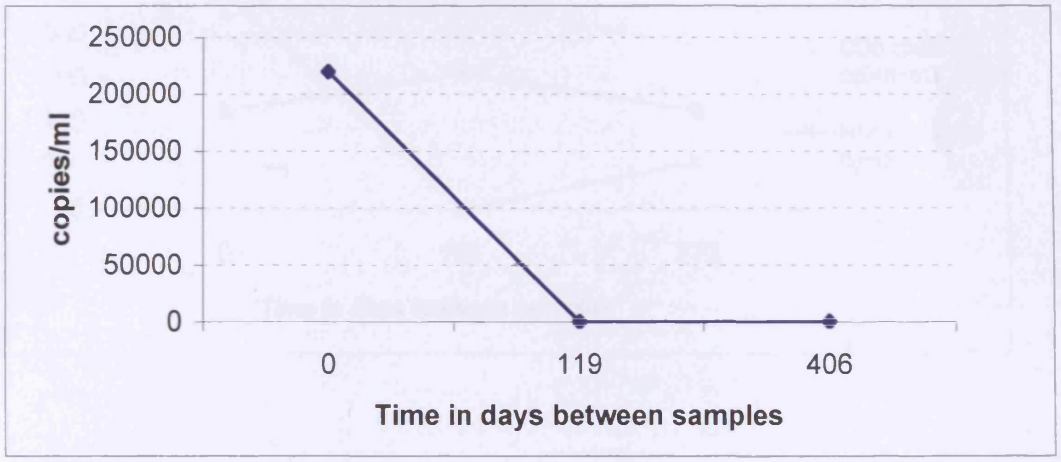


Figure 4.44: T Cell Changes; Patient 3

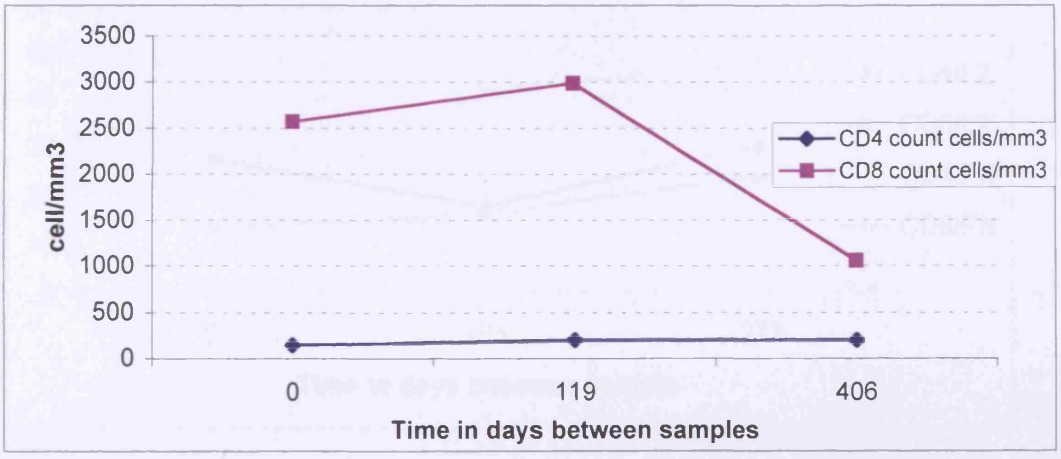


Figure 4.45: IL2 and IFNγ Changes; Patient 3

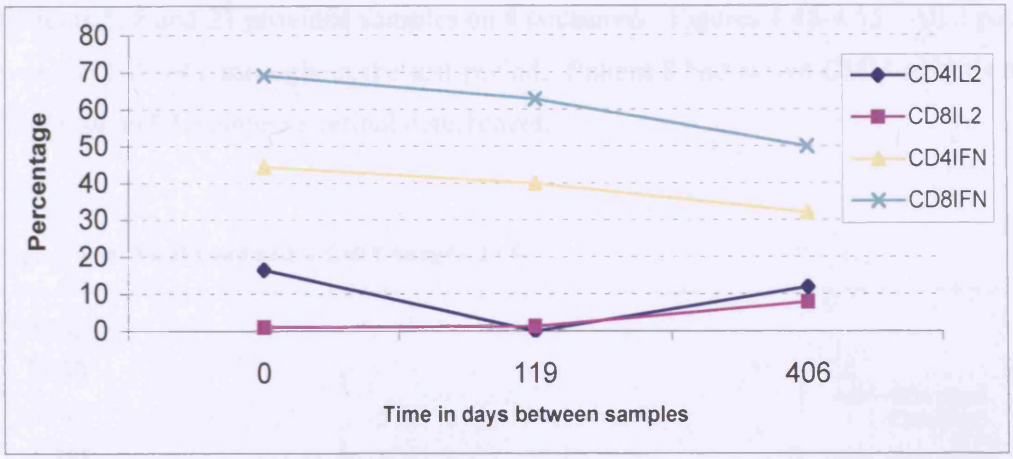


Figure 4.46: Viral Load and T Cell Changes; Patient 20

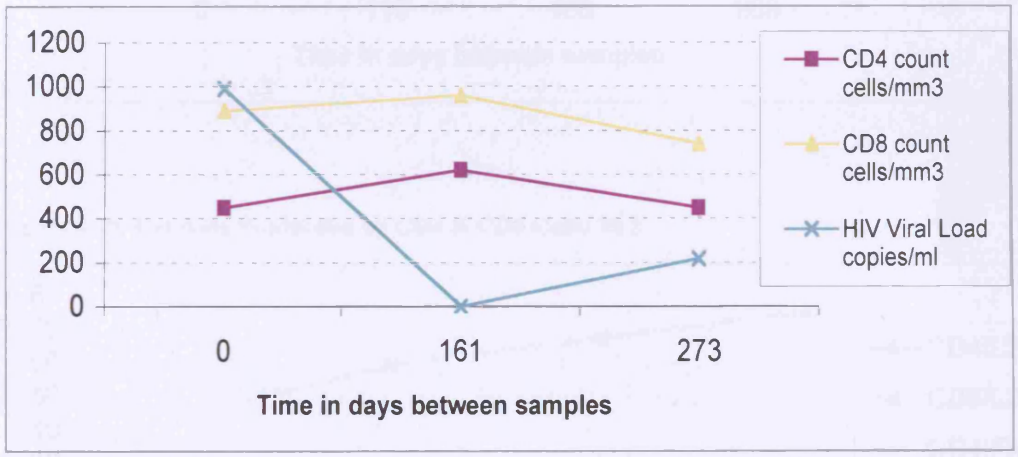
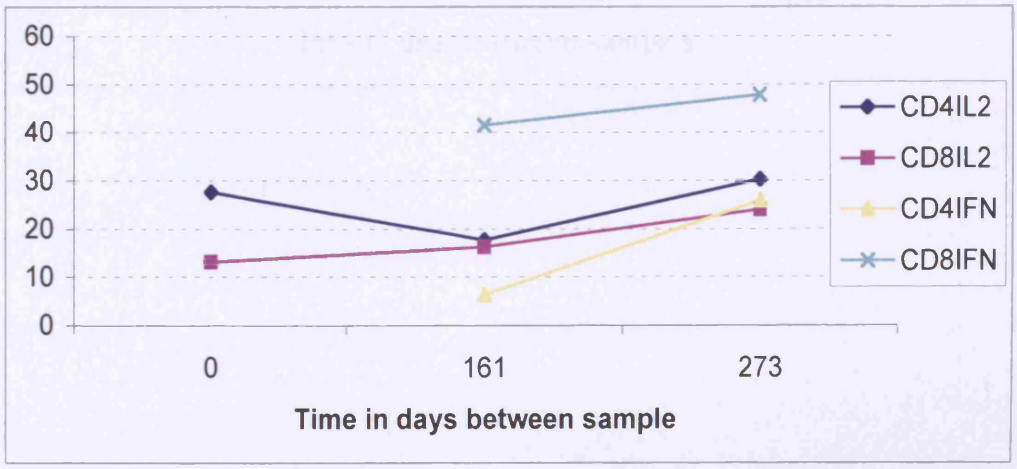


Figure 4.47: IL2 and IFNγ Changes; Patient 20



4.4.10.3 4 Visits

Patients 5, 8 and 21 provided samples on 4 occasions. Figures 4.48-4.55. All 3 patients were on HAART throughout the test period. Patient 8 had active CMV retinitis at the first visit and developed a retinal detachment.

Figure 4.48: Viral Load and T Cell Changes; Pt 5

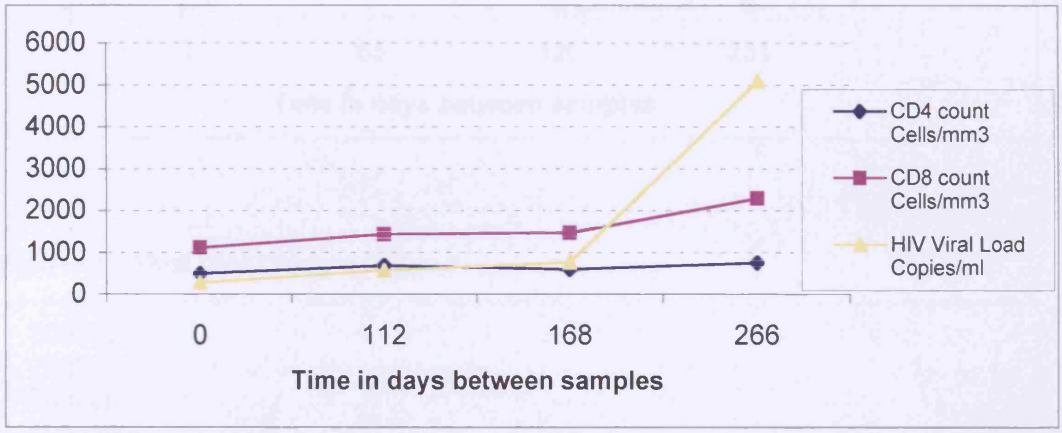


Figure 4.49: Cytokine Production by CD4 & CD8 Cells; Pt 5

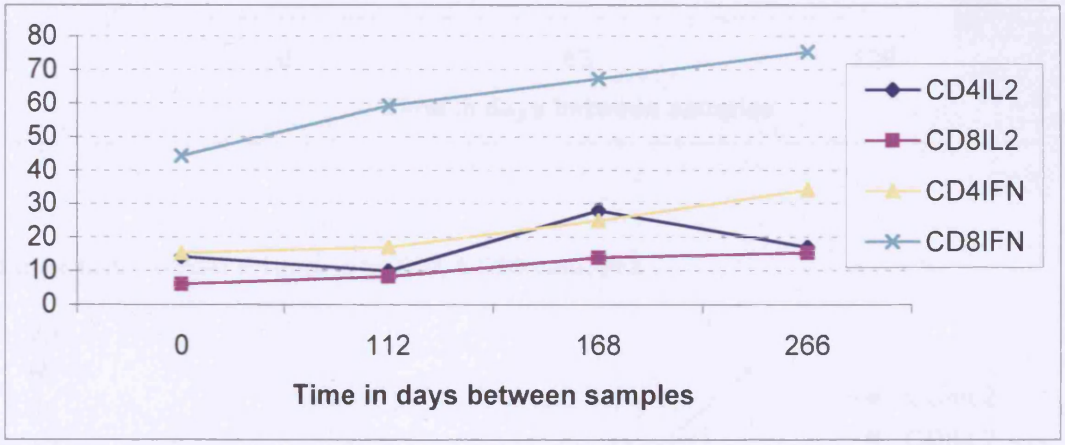


Figure 4.50: T Cell Changes; Patient 8

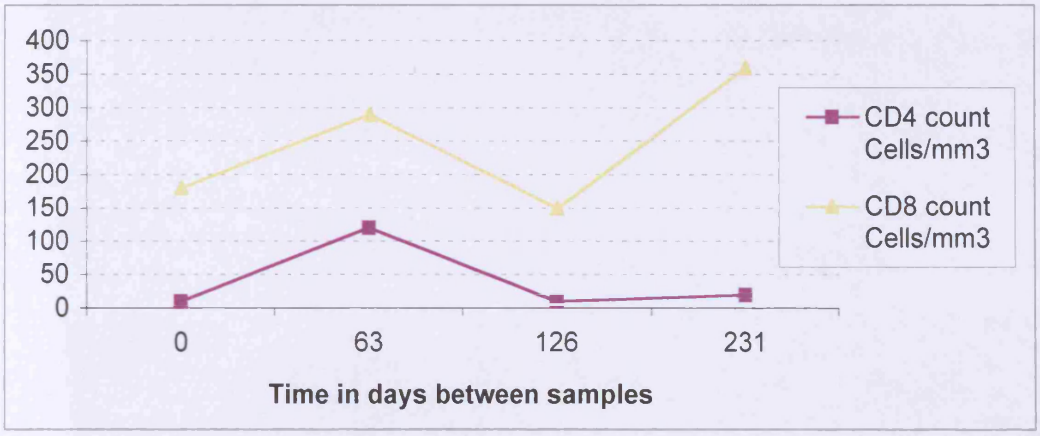


Figure 4.51: Viral Load Changes; Patient 8

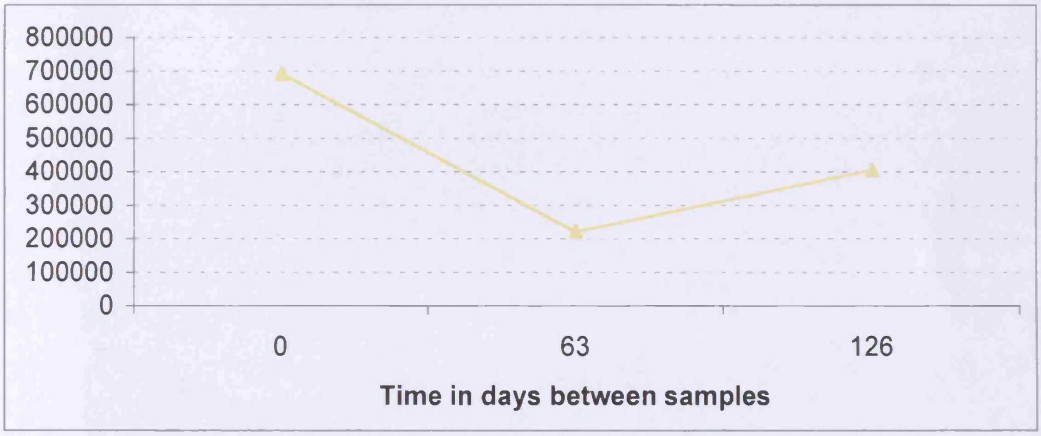


Figure 4.52: Cytokine Production by CD4 & CD8 Cells; Pt 8

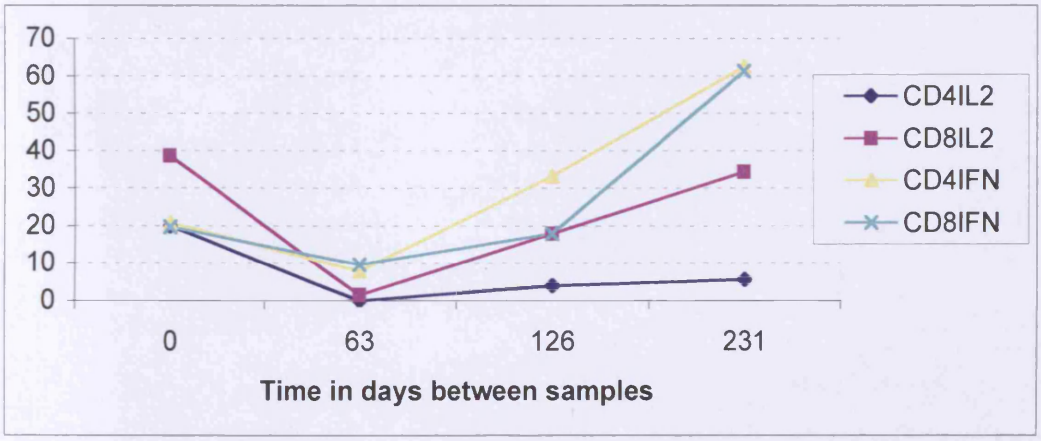


Figure 4.53: T Cell Changes; Patient 21

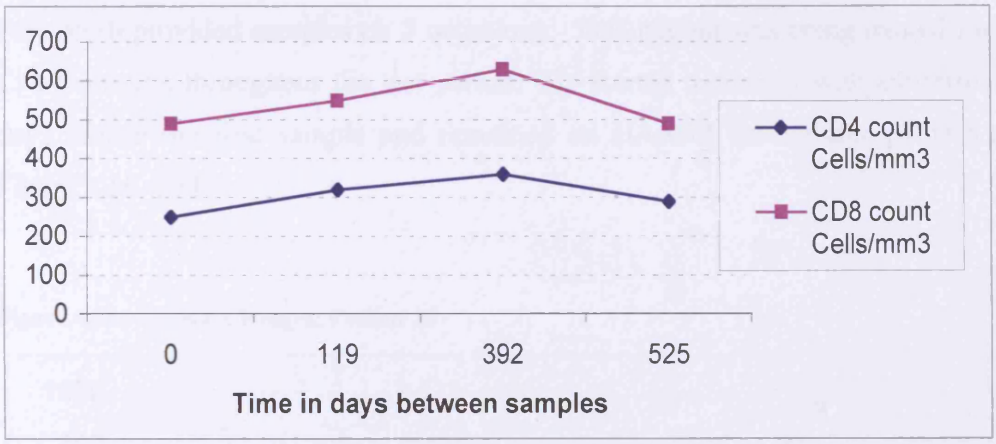


Figure 4.54: Viral Load Changes; Patient 21

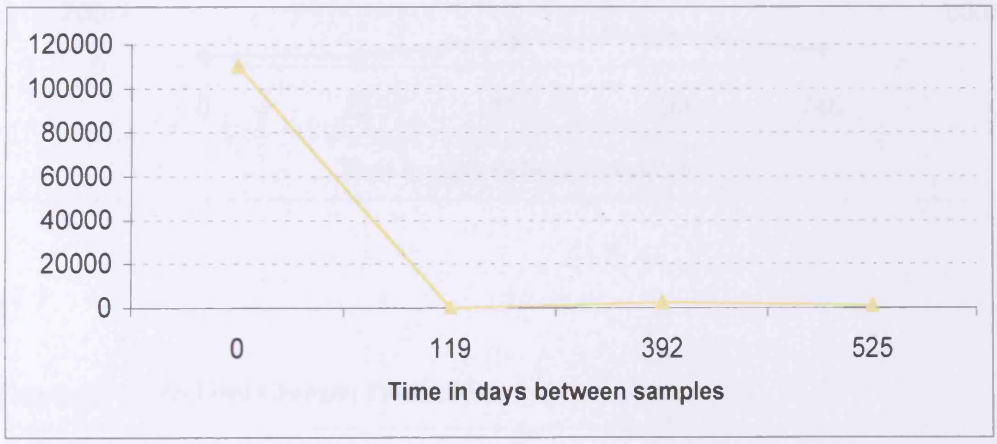
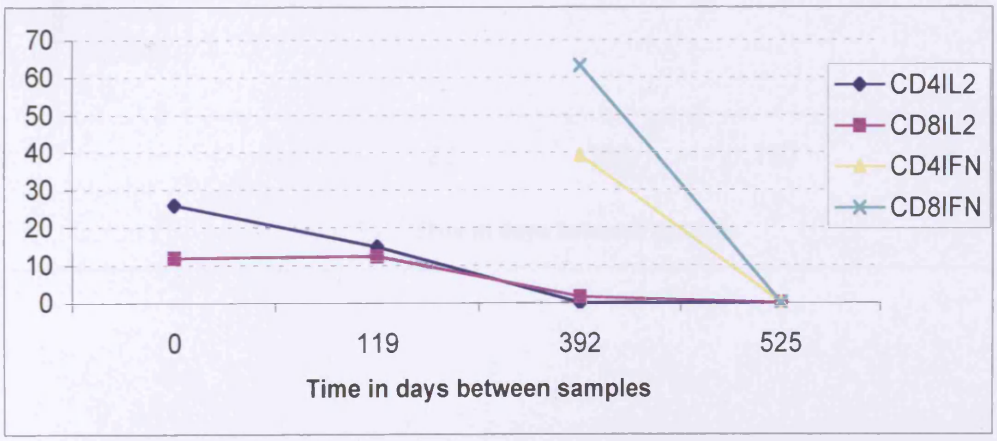


Figure 4.55: Cytokine Production by CD4 and CD8 Cells; Pt 21



4.4.10.4 5 Visits

Patient 30 provided samples on 5 occasions. This patient was being treated for active CMV retinitis throughout the test period. He started treatment with antiretrovirals 2 days before the first sample and remained on HAART throughout the test period. Figure 4.56-4.58.

Figure 4.56: T Cells Changes; Patient 30

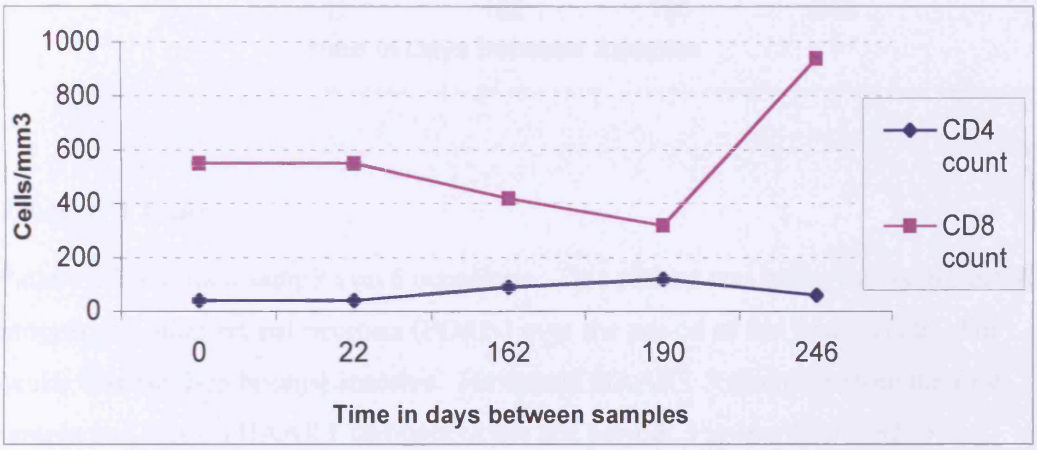


Figure 4.57: Viral Load Changes; Patient 30

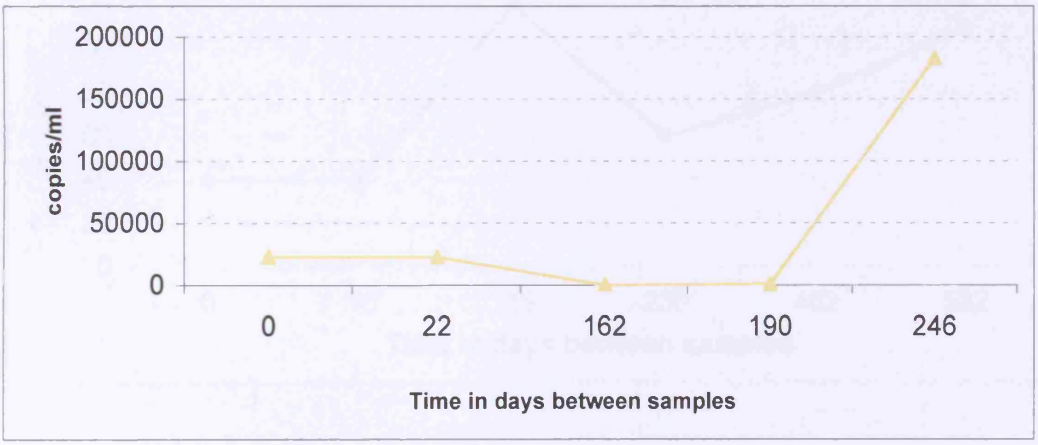
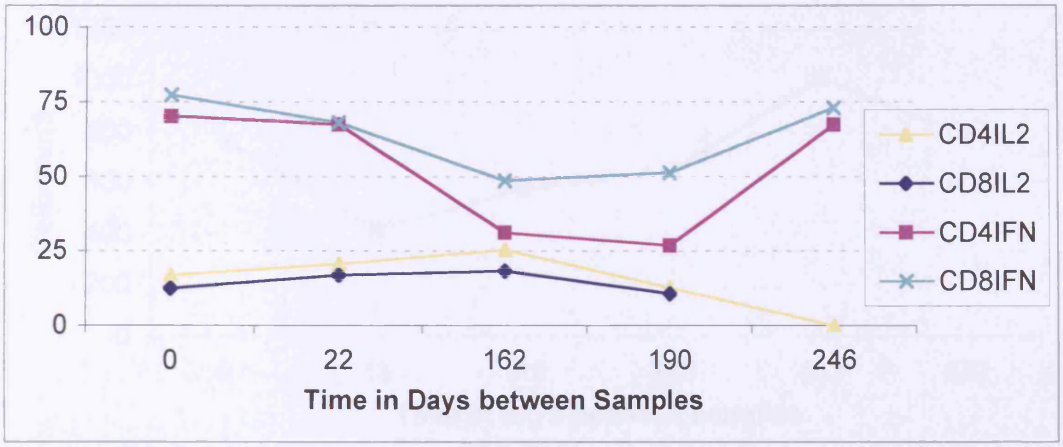


Figure 4.58: Cytokine Production by CD4 and CD8 Cells; Pt 30



4.4.10.5 6 Visits

Patient 19 provided samples on 6 occasions. This patient was being treated for active progressive outer retinal necrosis (PORN) over the period of the first 3 visits. The ocular disease then became inactive. He started HAART 3 months before the first sample and was on HAART throughout the test period. Figures 4.59-4.62.

Figure 4.59: CD4 Changes; Patient 19

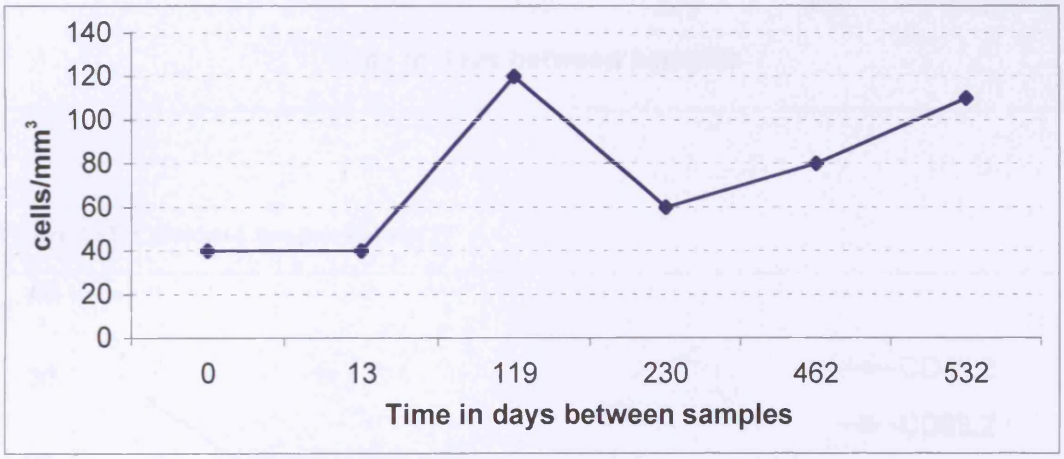


Figure 4.60: CD8 Changes; Patient 19

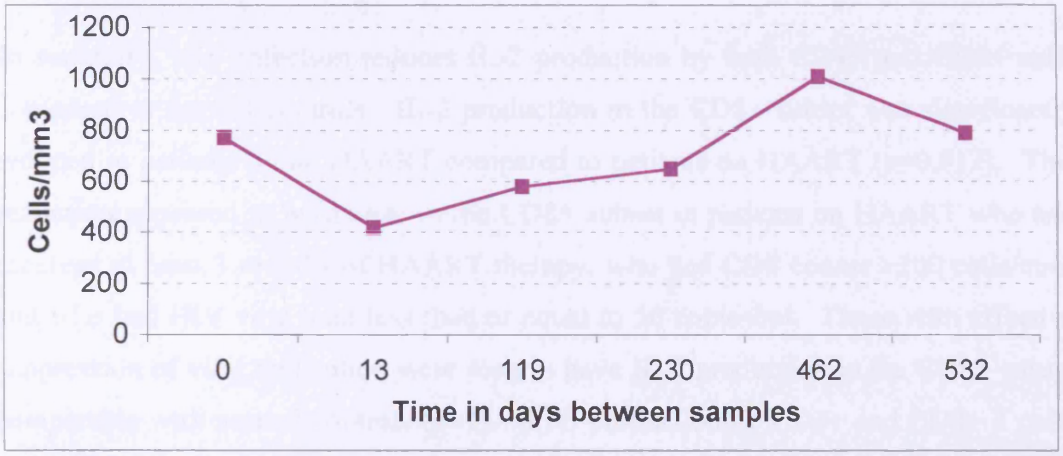


Figure 4.61: Viral Load Changes; Patient 19

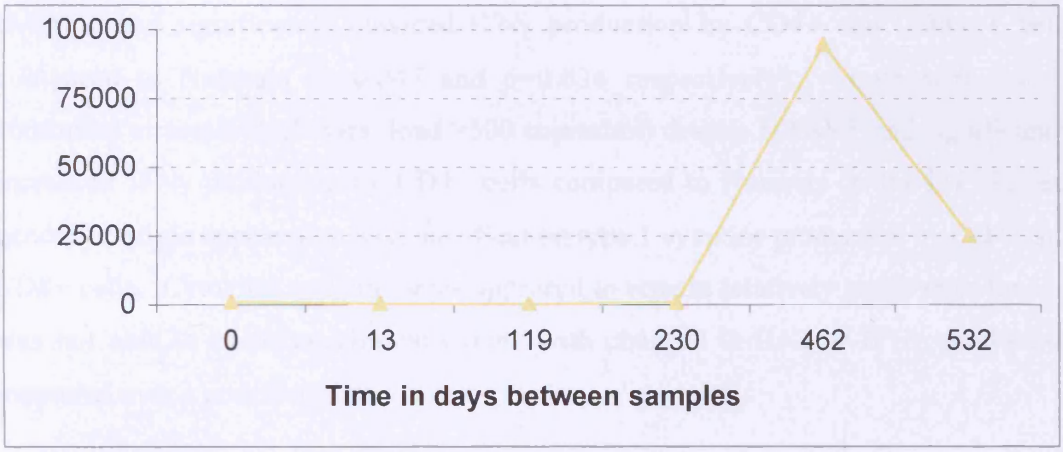
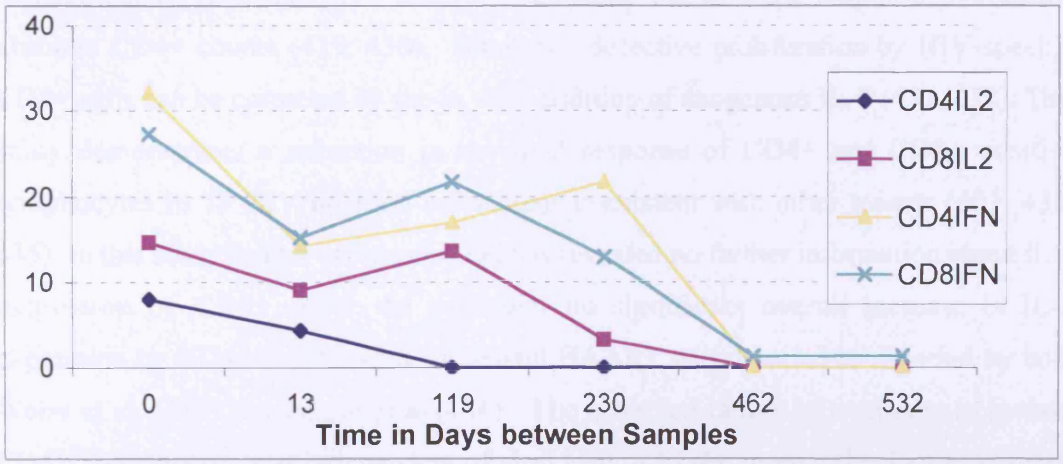


Figure 4.62: Cytokine Changes; Patient 19



4.5 SUMMARY

In summary, HIV infection reduces IL-2 production by both CD4+ and CD8+ cells compared to normal controls. IL-2 production in the CD8+ subset was significantly reduced in patients on no HAART compared to patients on HAART (p=0.012). This reduction appeared to normalize in the CD8+ subset in patients on HAART who had received at least 3 months of HAART therapy, who had CD4 counts >200 cells/mm^3 and who had HIV viral load less than or equal to 50 copies/ml. Those with effective suppression of viral replication were seen to have IL-2 production in the CD8+ subset comparable with normal controls (p=1). IFNγ production by CD4+ and CD8+ T cells was significantly increased in HIV patients compared to Normals (p=0.04 and p=0.02 respectively). No difference was seen in IFNγ production by CD4+ and CD8+ cells of HIV patients stable on no HAART compared to Normals. In contrast, patients on HAART had significantly elevated IFNγ production by CD4+ and CD8+ T cells compared to Normals (p=0.045 and p=0.036 respectively). Those with poorly controlled viraemia (HIV viral load >500 copies/ml) despite HAART had significantly increased IFNγ production by CD4+ cells compared to Normals (p=0.012). Patient gender or origin appeared to have no effect on type 1 cytokine production by CD4+ and CD8+ cells. Cytokine measurements appeared to remain relatively stable over time. I was not able to correlate clinical events with changes in IL-2 or IFNγ production measured over a period of time.

4.6 DISCUSSION

IL-2 is known to be central to the pathogenesis of HIV infection and treatment with supplementary IL-2 has been shown to enhance the immune response, increasing absolute CD4+ counts (429, 430). Similarly, defective proliferation by HIV-specific CD4+ cells can be corrected by the in vitro addition of exogenous IL-2 (431, 432). This study demonstrates a reduction in the IL-2 response of CD4+ and CD8+ positive lymphocytes in 73 HIV infected individuals consistent with other reports (403, 433-435). In this study further sub-group analysis revealed no further information about IL-2 expression by CD4+ cells. In particular no significant overall increase in IL-2 expression by CD4+ T cells with successful HAART was detected as reported by both Weiss et al. (433) and Imami et al.(436). The observed failure of treatment to reverse CD4+ T cell anergy was independent of viral load or CD4+ count. This lack of reversal

on HAART even in patients with significant immunological recovery as judged by an increased CD4 count of greater than 100 cell/mm^3 from nadir is in accord with the findings of Lederman et al(424) who reported that lymphocyte proliferation did not change significantly with time on antiretroviral therapy.

Sub-group analysis did reveal differences in IL-2 production in the CD8+ subset. This was significantly reduced in patients on no HAART compared to patients on HAART (p=0.012). Further sub-group analysis suggested that IL-2 production by CD8+ cells tended to normalize in patients on HAART who had received at least 3 months of HAART therapy, who had CD4 counts >200 cells/mm^3, who had a CD4+ change from nadir of more than100cells/mm^3, and who had HIV viral load less than or equal to 50 copies/ml. Those with effective suppression of viral replication were seen to have IL-2 production in the CD8+ subset comparable with normal controls (p=1). This improved CD8+ responsiveness occurred in patients with even very advanced immunodeficiency and low CD4+ counts.

These results add to the body of evidence suggesting the importance of CD8+ cells in the clinical functional immune recovery seen early in patients on HAART. IL-2 is known to reduce apoptosis and increase survival (437) of T-cells in HIV infection and this is happening at a time when CD4+T-cells are not showing improved function. Of interest is that changes in CD8+ IL-2 were seen in patients who had been on HAART for 3 months or more. It is known that HAART results in a rapid first phase (8-12week) increase in CD4+ and CD8+ T-cell numbers that likely represents redistribution from tissue sites(415, 416). Kempen et al report increased survival in patients with cytomegalovirus (CMV) retinitis who continued to have profound immunodeficiency despite receiving HAART(438). They defined immune recovery on HAART as a rise in CD4+ count rise by at least 50 cells/mm^3 from the time of CMV retinitis diagnosis to a level of > 100 cells/mm^3. They suggest that this reduction in mortality may have been due to immune recovery to a degree less than that included by their definition but sufficient to improve survival and/or control of HIV replication despite lack of immune recovery and/or benefits of HAART beyond those measured by its effects on CD4+ cell counts and HIV viral load measurements. The findings in the CD8+ subset in this study support benefits of HAART beyond those measured by the effects on CD4+ cell counts and HIV viral load measurements. Improvements in CD8+ function may occur sooner than those in CD4+ function and may be important at low CD4+ T cell counts.

In contrast the IFNγ data showed that IFNγ production by CD4+ and CD8+ T cells was significantly increased in HIV patients compared to Normals (p=0.04 and p=0.02 respectively). Patients on HAART also had significantly elevated IFNγ production by CD4+ and CD8+ T cells compared to Normals (p=0.045 and p=0.036 respectively). Sub-group analysis showed that those with poorly controlled viraemia (HIV viral load >500 copies/ml) despite HAART had significantly increased IFNγ production by CD4+ cells compared to Normals (p=0.012). Memory CD4+ T cells have been subdivided into central and effector memory cells based on their expression of surface antigens, their cytokine profiles and their ability to proliferate(439). Younes et al compared results from 10 viremic and 8 aviremic HIV-1 infected patients and found that persistent HIV generates a high frequency of IFNγ HIV-specific CD4+ T cells, lowers the frequency of IL-2 producing cells and interferes with the establishment and/or maintenance of long-lived memory(431). The findings presented here are consistent with this. Previous reports (397, 440) have shown increased IFNγ levels by ELISA in patients with high CD4+ counts with serum IFNγ levels falling as disease progressed. The observed fall in IFNγ levels with progressive disease may simply have been due to reduced cell numbers. The use of single cell analysis removes the problem of low cell numbers and demonstrates that the ability to produce IFNγ per se does not fall in advanced HIV disease. Klein et al. (403) reported a reduction in IFNγ expression in the CD4+ subset with progression to AIDS but in the CD8+ subset they saw an increase in IFNγ with progression which is consistent with our findings.

Our patient population was notably heterogenous with a large number of women, immigrants and patients presenting late in the course of their disease. There are published reports of CD4+ lymphocyte counts in HIV infection being different in men and women(441) but we were unable to demonstrate a gender difference in CD4+ lymphocyte function. Likewise no effect of ethnicity was seen in line with the previously reported UK study where similar rates of CD4+ lymphocyte decline were seen in HIV-1 infected Africans and non-Africans in London(442). These results suggest that the high proportion of women and African patients in our patient populations does not bias our results.

Analysis of repeat samples from individual patients suggest that although the test appears to be repeatable, results did not correlate well with other measures of clinical activity such as CD4 count, CD8 count or HIV viral load. The results suggest that IL-2

and IFNγ measurements are not good surrogate markers for monitoring the level of immunosuppression or the effect of HAART within individuals.

These studies measured the magnitude of bulk T lymphocyte responses to mitogen rather than the response of viral-specific sub-population of T cells. PMA+ionomycin was used in order to obtain the maximal level of T cell activation for each sample. This was considered important for reducing variability when comparing repeated measurements over time. It is, however, likely that the magnitude of CD4 and CD8 responses alone will not be as predictive of protection from disease as a combination of magnitude and other factors such as epitope breadth and affinity, phenotype, and function of antigen-specific cells(443). This can now be taken into account. Antigen specific responses can be obtained by using intact protein, viral lysates or peptides. MHC-peptide oligomers that require known peptide sequences matched to specific class 1 or class II MHC can be used and MHC class I restricted tetramer complexes provide information on peptide specific CD8 responses. In general peptides containing 15 amino acid residues each, and overlapping by 11 amino acid residues elicit equivalent CD4 responses to the intact proteins from which the peptide sequences were derived(444). These 15 amino acid peptides theoretically contain every single T cell epitope and can be used independently of the patient's MHC haplotype. When protein antigens are used an additional incubation period in the absence of brefeldin A is performed to allow for optimal antigen processing and presentation.

This study has the advantage of quantifying lymphocyte cytokine responses at the single cell level and relating this to clinical parameters. It confirms functional defects in T cell function in HIV infected patients on HAART compared to Normals that are clearly quantifiable even in the setting of higher T cell numbers or a good response to therapy. Despite single cell intracellular cytokine analysis and good patient numbers the data does not support the theory that a simple Th1 to Th2 switch, reversing on HAART therapy, is responsible for the cytokine changes occurring in HIV infection.

CHAPTER 5

OVERALL CONCLUSIONS

AND

SUGGESTED FURTHER RESEARCH

The potential for cytokine production on a single cell basis in HIV positive patients with different clinical profiles and patients receiving treatment for autoimmune disease was assessed. PMA and ionomycin were chosen as a potent stimulus for T-cell activation that would maximize cytokine expression. Maximal stimulation was chosen to avoid the possibility for variable results that may be seen with submaximal stimuli.

Despite exquisite sensitivity measurement of IL-2 or IFNγ by intracellular staining and flow cytometry does not appear to correlate well with clinical profiles or drug regimes in autoimmune uveitis. Whilst perhaps not surprising in those who have purely ocular disease analysis also showed no relationship in patients who had autoimmune uveitis on a background of systemic disease such as Behcet's. This is in contrast to other published reports. Difficulties in categorizing patients according to activity may have been responsible for masking an effect. Visual acuity and vitreous haze are considered the best indicators of activity but are markedly affected by media opacities. These indices also become much less helpful in chronic uveitis when chronic macular oedema, epiretinal membranes or ischaemia are more common. In these situations the patients symptoms are often the best indicator of activity and perhaps patient scoring of activity would have been helpful. The ocular survey of ocular disease in immunosuppressed patients highlights the importance of patient symptoms for guiding further investigation. Systemic treatment with drugs known to modulate Th1 cytokine production would have been expected to show demonstrable changes in cytokine production. Such changes were seen for IL-2 production in patients on CsA regimes. The changes seen in these patients were of the on-off type and were not titratable against dose of CsA.

Difficulties in seeing an effect may be due to problems with the power of the study although the numbers of patients tested was high compared to other studies of a similar type. Also the statistics used are deliberately conservative to limit the risks of finding spurious significance with the large number of comparisons. The complexity of the immune response itself, however, with cytokine networks upregulating and down regulating in response to minute changes in the intercellular environment makes interpretation difficult. Better interpretation of the data would be possible with a clearer understanding of what the normal variations are. This could be obtained by taking repeated samples from a larger number of healthy subjects over a year to document the effect of repeat testing and intercurrent infections.

Repeat samples were taken to give an impression of changes in cytokine profile over time in HIV and uveitis patients on treatment. Due to the large amount of patient variables this data was not analyzed statistically but each patient is presented here as a case study. This provides interesting information about the individuals with elevated IFNγ being associated with immune activation but trends could not be seen that can be extrapolated to the general patient populations.

Analysis of the data that I do have is also limited by the fact that the patient groups were complex and patients were on a wide range of disease modifying therapies. Analysis of more homogenous patient groups would provide a clearer idea of the effect of disease on IL-2 and IFNγ. Virologically HIV has three distinct phases: conversion, immune depletion and AIDS. In AIDS frequent intercurrent infections will result in immune activation and thus complicate the picture. Chronic immune activation is probably responsible for the high levels of IFNγ seen in our patients. Testing of patients before the initiation of systemic medications would have given a clearer idea of the effect of disease on IL-2 and IFNγ so that the added effect of disease modifying therapies could be more clearly seen. Analysis of uveitis patients who are managed with periocular or intraocular steroids would provide a useful negative control group.

The holy grail remains a surrogate marker that could be used to quantify the level of immunosuppression. Although cytokines can be accurately measured and can be quantified, because the immune system is highly complex and highly responsive effectors of the immune response may not be stable enough to be good candidates for assessing clinical progress. Further investigation of cytokine profiles would be interesting for the information it provides about disease processes and treatment. This study does not, however, support the use of IL-2, IFNγ to guide clinical practice in the highly complex system that is the patient. Instead, it emphasizes the need for careful history and examination. Likewise, the ocular survey in transplant patients highlighted the value of symptoms in reducing unnecessary screening.

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